Biomarkers in Disease:
Methods, Discoveries and Applications
Series Editors: Vinood B. Patel
Victor R. Preedy

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Vinood B. Patel · Victor R. Preedy
Rajkumar Rajendram
Editors

Biomarkers in Toxicology

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Biomarkers in Disease: Methods, Discoveries and Applications

In the past decade there has been a major sea change in the way disease is diagnosed and investigated due to the advent of high throughput technologies, such as microarrays, lab on a chip, proteomics, genomics, lipomics, metabolomics etc. These advances have enabled the discovery of new and novel markers of disease relating to autoimmune disorders, cancers, endocrine diseases, genetic disorders, sensory damage, intestinal diseases etc. In many instances these developments have gone hand in hand with the discovery of biomarkers elucidated via traditional or conventional methods, such as histopathology or clinical biochemistry. Together with microprocessor-based data analysis, advanced statistics and bioinformatics these markers have been used to identify individuals with active disease or pathology as well as those who are refractory or have distinguishing pathologies. New analytical methods that have been used to identify markers of disease and is suggested that there may be as many as 40 different platforms. Unfortunately techniques and methods have not been readily transferable to other disease states and sometimes diagnosis still relies on single analytes rather than a cohort of markers. There is thus a demand for a comprehensive and focused evidenced-based text and scientific literature that addresses these issues. Hence the formulation of Biomarkers in Disease. The series covers a wide number of areas including for example, nutrition, cancer, endocrinology, cardiology, addictions, immunology, birth defects, genetics and so on. The chapters are written by national or international experts and specialists.

Series Titles

1. General Methods in Biomarker Research and Their Applications
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3. Biomarkers in Cardiovascular Disease
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5. Biomarkers in Bone Disease
6. Biomarkers in Liver Disease
7. Biomarkers in Diabetes
8. Biomarkers in Nutrition
9. Biomarkers in Toxicology
10. Biomarkers in Trauma, Injury and Critical Care

Vinood B. Patel • Victor R. Preedy •
Rajkumar Rajendram
Editors

Biomarkers in Toxicology

With 175 Figures and 79 Tables

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Preface

In recent years, there have been major changes in the way diseases are diagnosed and investigated due to the advent of high-throughput technologies and advances in chemistry and physics. This has led to the development of microarrays, lab-on-a-chip, proteomics, genomics, lipomics, metabolomics, and other new platforms. These advances have enabled the discovery of new and novel markers of disease relating to autoimmune disorders, cancers, endocrine diseases, genetic disorders, sensory damage, intestinal diseases, and many other conditions too numerous to list here. In many instances, these progressions have gone hand in hand with analysis of biomarkers elucidated via traditional methods, such as histopathology, immunoassays, and clinical biochemistry. Together with microprocessor-based data analysis, advanced statistics, and bioinformatics, these markers have been used to identify individuals with active disease as well as those who are refractory or have distinguishing pathologies.

Unfortunately, techniques and methods have not been readily transferable to other disease states, and sometimes diagnosis still relies on a single analyte rather than a cohort of markers. Furthermore, the discovery of many new markers has not been put into clinical practice partly because of their cost and partly because some scientists are unaware of their existence or the evidence is at the preclinical stage. There is thus a demand for a comprehensive and focused evidence-based text that addresses these issues. Hence the book ***Biomarkers in Disease: Methods, Discoveries and Applications – Biomarkers in Toxicology***. It imparts holistic information on the scientific basis of health and biomarkers and covers the latest knowledge, trends, and links with treatments. It links conventional approaches with new platforms.

In the present book, ***Biomarkers in Toxicology*** we have sections on:

1. ***Single components***
2. ***Panels and arrays***
3. ***Genetic, cellular, and histological variables***
4. ***Functional and physiological variables and platforms***
5. ***Biomarkers in specific conditions or scenarios***
6. ***Biomarkers in models and modeling toxicity***
7. ***Resources***

The ability to transcend the intellectual divide is aided by the fact that each chapter has:

- ***Key Facts (areas of focus explained for the layperson)***
- ***Definitions of Words and Terms***
- ***Applications to Prognosis, Other Diseases, or Conditions***
- ***Summary Points***

The material in ***Applications to Prognosis, Other Diseases, or Conditions*** pertains to speculative or proposed areas of research, cross-transference to other diseases or stages of the disease, translational issues, and other areas of wide applicability.

The Editors recognize the difficulties in assigning chapters to parts of the book, as some chapters can fit into more than one section. Nevertheless, the book has enormously wide coverage and is well indexed.

The chapters are written by national and international experts. This book is designed for toxicologists, pharmacologists, clinical biochemists, health scientists specializing in drugs of abuse, epidemiologists, researchers, doctors, and nurses, from students to practitioners at the higher level. It is also designed to be suitable for lecturers and teachers in health care and academic libraries as a reference guide.

London, UK — Vinood B. Patel
London, UK — Victor R. Preedy
Riyadh, Saudi Arabia — Rajkumar Rajendram
January 2023

Series Preface

In the past decade there have been major changes in the way diseases are diagnosed and investigated due to the advent of high-throughput technologies and advances in chemistry and physics. This has led to the development of microarrays, lab-on-a-chip, proteomics, genomics, lipomics, metabolomics, and other new platforms. These advances have enabled the discovery of new and novel markers of disease relating to autoimmune disorders, cancers, endocrine diseases, genetic disorders, sensory damage, intestinal diseases and many other conditions too numerous to list here. In many instances these developments have gone hand in hand with analysis of biomarkers elucidated via traditional methods, such as histopathology, immunoassays and clinical biochemistry. Together with microprocessor-based data analysis, advanced statistics and bioinformatics these markers have been used to identify individuals with active disease as well as those who are refractory or have distinguishing pathologies.

Unfortunately, techniques and methods have not been readily transferable to other disease states, and sometimes diagnosis still relies on a single analyte rather than a cohort of markers. Furthermore, the discovery of many new markers has not been put into clinical practice partly because of their cost and partly because some scientists are unaware of their existence or the evidence is at the preclinical stage. There is thus a demand for a comprehensive and focused evidenced-based text that addresses these issues. Hence the book series **Biomarkers in Disease: Methods, Discoveries and Applications**. It imparts holistic information on the scientific basis of health and biomarkers and covers the latest knowledge, trends and treatments. It links conventional approaches with new platforms. The ability to transcend the intellectual divide is aided by the fact that each chapter has:

- ***Key Facts (areas of focus explained for the lay person)***
- ***Definitions of Words and Terms***
- ***Potential Applications to Prognosis, Other Diseases or Conditions***
- ***Summary Points***

The material in ***Potential Applications to Prognosis, Other Diseases or Conditions*** pertains to speculative or proposed areas of research, cross-transference to

other diseases or stages of the disease, translational issues, and other areas of wide applicability.

The Series is expected to prove useful for clinicians, scientists, epidemiologists, doctors and nurses, and also academicians and students at an advanced level.

The Editors

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About the Editors

Dr. Vinood B. Patel, BSc, PhD, FRSC, is a Reader in Clinical Biochemistry at the University of Westminster. Dr. Patel graduated from the University of Portsmouth with a degree in Pharmacology and completed his PhD in protein metabolism from King's College London in 1997. His postdoctoral work was carried out at Wake Forest University Baptist Medical School studying structural-functional alterations to mitochondrial ribosomes, where he developed novel techniques to characterize their biophysical properties. In 2014, he was elected as a Fellow to the Royal Society of Chemistry. Dr. Patel is a nationally and internationally recognized researcher and was involved in several NIH-funded biomedical grants related to disease. Dr. Patel has extensively published biomedical books in the area of diet, biomarkers, toxicology, nutrition, and health prevention.

Professor Victor R. Preedy, BSc, PhD, DSc, FRSB, FRSPH, FRCPath, FRSC, is Professor of Clinical Biochemistry at King's College Hospital, Emeritus Professor of Nutrition Biochemistry at King's College London, and Visiting Professor at the University of Hull. Professor Preedy graduated in 1974 with an Honors Degree in Biology and Physiology with Pharmacology. He gained his PhD from the University of London in 1981. In 1992, he received his Membership of the Royal College of Pathologists, and in 1993 he gained his second doctoral degree for his outstanding contribution to protein metabolism in health and disease. Professor Preedy was elected as a Fellow to the Institute of Biology in 1995 and to the Royal College of Pathologists in 2000. Since then he has been elected as a Fellow to the Royal Society for the Promotion of Health (2004) and the Royal Institute of Public Health (2004). In 2009, Professor Preedy became a Fellow of the Royal Society for Public Health and in 2012 a Fellow of the Royal Society of Chemistry. In his career, Professor Preedy has carried out research at the Cardiothoracic Institute, National Heart Hospital (part of Imperial College London), The School of Pharmacy (now Part of University College London), and the MRC Centre at Northwick Park Hospital. He has collaborated with research groups in Finland, Japan, Australia, USA, and Germany. He is a leading expert on the science of health and has a long-standing interest in biomarkers for over 30 years, especially related to tissue pathology. He has lectured nationally and internationally. To his credit, Professor Preedy has published over 700 articles, which includes peer-reviewed manuscripts based on

original research, abstracts and symposium presentations, reviews, and numerous books and volumes.

Dr. Rajkumar Rajendram, AKC BSc (Hons) MBBS (Dist) MRCP (UK) EDIC FRCA FFICM FRCP Edin FRCP Lon, is a clinician scientist who has gained extensive experience in toxicology during his practice in anesthesia, intensive care, and acute medicine. Dr. Rajendram graduated in 2001 with a distinction from Guy's, King's, and St. Thomas' School of Medicine in London. As an undergraduate he was awarded several prizes, merits, and distinctions in pre-clinical and clinical subjects.

Dr. Rajendram began his postgraduate medical training in general medicine and intensive care in Oxford. He attained membership of the Royal College of Physicians (MRCP) in 2004 and completed specialist training in acute and general medicine in Oxford in 2010. Dr. Rajendram also trained in anesthesia and intensive care in London. He obtained extensive experience in the management of intoxication at the internationally renowned critical care centers of the Oxford University Hospitals, Royal Free London Hospitals, Royal London Hospital, and the University College Hospital, London. He was awarded fellowship of the Royal College of Anaesthetists (FRCA) in 2009 and fellowship of the Faculty of Intensive Care Medicine (FFICM) in 2013. He was awarded the European diploma of intensive care medicine (EDIC) in 2014. In 2017, he became a fellow of the Royal College of Physicians, Edinburgh, and then became a fellow of the Royal College of Physicians, London, in 2019.

Dr. Rajendram returned to Oxford as a Consultant in Acute General Medicine at the John Radcliffe Hospital, Oxford, before moving to the Royal Free London Hospitals as a Consultant in Intensive Care, Anesthesia, and Peri-operative Medicine. He is currently a Consultant in Internal Medicine at King Abdulaziz Medical City, National Guard Heath Affairs, Riyadh, Saudi Arabia, where he regularly manages victims of intoxication.

As a clinician scientist, Dr. Rajendram has also devoted significant time and effort into research and education. He is a joint appointment Assistant Professor in the College of Medicine, King Saud bin Abdulaziz University for Health Sciences, Riyadh, Saudi Arabia, and has published over 300 textbook chapters, review articles, peer-reviewed papers, and abstracts many of which are related to the pathogenesis, investigation, and treatment of disease which includes critical care and alcohol-induced toxicity.

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Part I

Single Components

1 Soluble Guanylyl Cyclase Alpha1 Subunit as a Biomarker of Toxicity: Applications to Investigate Endocrine-Disrupting Chemicals

María Teresa Pino and Jimena Paula Cabilla

Contents

Abstract

Endocrine-disrupting chemicals (EDCs) are a growing group of naturally occurring and synthetic substances in the environment that negatively impacts human and wildlife health. Most persist in water and soil for many years, being able to bioaccumulate and act at very low doses. Xenoestrogens, a subgroup of EDCs, can mimic estrogen actions directly related with endocrine disorders and cancer.

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Identification and characterization of these compounds by several toxicity assays are increasingly important. The present work discusses current detection methods of estrogen-like activities of EDCs and reviews the advantages of including new markers of exposure such as soluble guanylyl cyclase alpha1 subunit (sGCα1) in in vitro assays.

Keywords

Soluble guanylyl cyclase alpha1 subunit · In vitro toxicity assays · Endocrine-disrupting chemicals · Estrogen receptor · Xenoestrogens · Dose-response · Marker of exposure · Screening methods · Environment · Cell lines

Abbreviations

E2	17β-estradiol
EDC	Endocrine-disrupting chemicals
ER	Estrogen receptor
ERE	Estrogen-response element
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
IGF-1R	Insulin growth factor 1 receptor
NO	Nitric oxide
sGC	Nitric oxide-sensitive or soluble guanylyl cyclase
sGCα1	Soluble guanylyl cyclase alpha1 subunit
VTG	Vitellogenin
XE	Xenoestrogens

Introduction

Endocrine-disruptor compounds were first described by Theo Colborn and colleagues in 1991; since then, many investigations are being conducted to identify chemicals potentially dangerous for health. Identification and characterization of substances with endocrine disrupting effects pave the way to establish or update policies about limiting or banning the use of these compounds. In this sense, international organizations and governments have established batteries of in vitro and in vivo assays which are periodically revised and upgraded to improve the study of these substances. In vitro studies focus on the mechanisms of action while in vivo assays address mainly biological effects. Soluble guanylyl cyclase alpha 1 subunit raised as a highly estrogen-responsive protein under different physiological and experimental conditions. Here we review the main topics regarding endocrine-disruptor compounds study, from their diversity and sources, to current batteries of assays and the advantage of including a suitable marker of exposition as soluble guanylyl cyclase alpha1 subunit.

Endocrine-Disruptor Compounds (EDCs)

Wildlife and human exposure to environmental contaminants has raised concern due to their adverse impact on health. Endocrine disruptor compounds (EDCs) are a continuously growing group of exogenous substances that affect the endocrine system by causing negative health effects in an organism and/or its progeny (Diamanti-Kandarakis et al. 2009; Zoeller et al. 2012; Gore et al. 2015). While they are mostly anthropogenic compounds or their mixtures, many naturally occurring compounds such as toxins and hormones from plants and fungi are also included in this group. EDCs are ubiquitously found in food and food packaging, water, cosmetic products, household goods, plastic ware, detergents, fabrics and upholstery, solvents, electronics, flame retardants, medical equipment, ambient air, fungicides, pesticides, and pharmaceutical products (Gore et al. 2015). Even when many pharmaceuticals are designed to target the endocrine system for therapeutic purposes, release of these drugs and/or their metabolites into waterways and sewage systems contributes to contaminate the environment, also potentially leading to endocrine disruption. Depending on their chemical structure, many EDCs have long half-lives and the ability to bioaccumulate, thereby biomagnifying in the food chain as well as in soil and water. The use of many of these compounds has been banned; nevertheless, due to their lengthy persistence, they are still present in the environment at relevant concentrations. Some representative EDCs and their binding affinities are provided in Fig. 1.

Numerous EDCs, including the so-called metabolic disrupting compounds (MDCs), tend to accumulate in adipose tissue and liver, therefore possessing continued potential for adverse health effects. Under different physiological conditions involving mobilization of fat stores (e.g., stress, fasting, pregnancy, and lactation), they are released into circulation, making them rapidly bioavailable (Heindel et al. 2017; Papalou et al. 2019).

In its most comprehensive form, EDC is "an exogenous chemical or mixture of chemicals that interferes with any aspect of hormone action" (Zoeller et al. 2012). More specifically, EDC is capable of interfering with the endocrine system at several points, from impeding synthesis, secretion, transport, distribution, metabolism, binding, or elimination of natural hormones, to mimicking hormone action at the level of receptor binding and signal transduction, thereby altering homeostasis of the body and contributing to adverse outcomes (La Merrill et al. 2020). Exposition to EDCs may alter physiology during an individual's whole life span, from fetal development to adulthood, this adverse effect being dependent on timing and dose exposure (Diamanti-Kandarakis et al. 2009). Moreover, some EDCs were shown to go further by causing epigenetic changes leading to effects on several organs (Christensen and Marsit 2011; Rissman and Adli 2014).

A large body of evidence support the significant role of EDCs in the development of many diseases, such as reproductive problems, endocrine-related cancers, neurodevelopmental disorders, immune-related and metabolic diseases

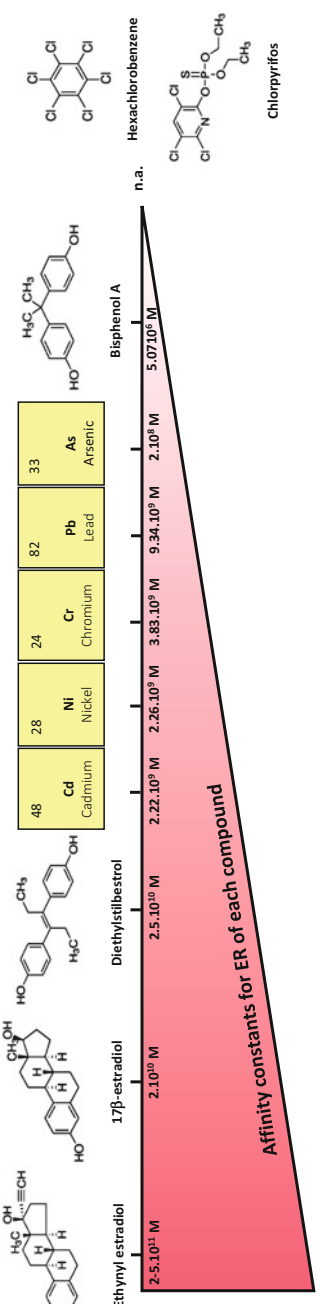

Fig. 1 Structure and affinity constants for ER of E2 and some EDCs with estrogenic activity. A heterogenic group of EDCs can exhibit estrogenic activity despite having no affinity to ER or structural similarity to E2 (von Schoultz et al. 1989; Kuiper et al. 1997; Martin et al. 2003)

(UNEP/WHO et al. 2013; Gore et al. 2015). Most humans and wildlife are continually exposed to these harmful compounds, as observed in the increasing incidence of many endocrine-related disorders and global rates of endocrine-related cancers.

Estrogens

Estrogen is an extensive term used to describe the predominant female hormone. Three major estrogen derivatives are known: 17β-estradiol (E2) is the most potent and abundant form during reproductive age, whereas estrone (E1) is the primary form of estrogen after menopause, and estriol (E3) is a by-product of its metabolism (Kiyama and Wada-Kiyama 2015; Fuentes and Silveyra 2019). All of these are able to bind estrogen receptors (ER) and exert their actions at very low concentrations (Chamkasem and Toniti 2015). Given the physiological relevance and predominance of E2 during reproductive life, estrogens are usually referred as E2. E2 are primarily synthesized in the ovaries, but also in the adrenal glands and adipose tissue and exert a plethora of key actions in human organisms. They are involved in many physiological processes such as the regulation of menstrual reproductive cycles, physiology of reproductive organs – breast, endometrium, and ovary – and immune system, lipid metabolism, protein synthesis, as well as in pathological processes, such as the onset and progression of several diseases (e.g., cancer and neurodegenerative, cardiovascular and autoimmune diseases) (Kiyama and Wada-Kiyama 2015; Benagiano et al. 2019; Merrheim et al. 2020).

E2 are able to freely trespass cell membrane and bind E2 receptors (ER) located in the cytoplasm and nucleus (Yaşar et al. 2017). Two major isoforms of ER have been described: ERα, involved in well-known proliferative actions, and ERβ, which counteracts most ERα actions, e.g., cell proliferation in tissues such as breast and uterus (Paterni et al. 2014). Upon binding E2, the receptor-hormone complex acts as a transcription factor by binding specific DNA sequences located in the promoter regions of target genes: the E2 responsive elements (EREs), which constitute canonical ER signalling. Furthermore, ER can interact with other transcription factors, such as Sp1 and AP-1, which in turn bind non-ERE genes (Ikeda et al. 2015). Moreover, ER can display noncanonical actions by acting at plasma membrane level and activating different signalling pathways such as PI3K and MAPK (Prossnitz and Barton 2011; Ranganathan et al. 2019). E2 circulating levels are strictly controlled by the hypothalamic-pituitary-gonadal axis through negative feedback. These mechanisms classically involve inhibition of E2 synthesis and release from ovary, as well as decreased release of gonadotropin hormone-releasing hormone (GnRH) and gonadotropins (LH and FSH) at hypothalamic and pituitary levels, respectively. At cellular level, many other negative feedbacks tend to avoid E2 overstimulation, such as downregulation of active, full-length ER (ERα66) and upregulation of shorter ER isoforms (ERα36 and ERα46) which shields lower or no transcriptional activity compared to EREα66, thereby acting as decoy receptors (Mitchner et al. 1998; Penot et al. 2005; Wang et al. 2006), and many ERα-splicing variants in uterus (Varayoud et al. 2005).

Xenoestrogens

One group of EDCs receives particular attention from the scientific community: the so-called xenoestrogens (XEs) or E2-like compounds. They are considered the major disruptors of the endocrine system. This group is composed of a great variety of both natural and synthetic chemicals varying in structure but sharing the common feature of mimicking or blocking natural E2 actions (Li et al. 2013; Paterni et al. 2017). As seen in Fig. 1, many XEs have little or no resemblance to E2; therefore, the potential XE activity of a compound cannot be predicted a priori from its chemical structure.

XEs can be divided into two groups: natural, comprising phytoestrogens and mycoestrogens; and synthetic, including those with pharmaceutical and industrial use, food preservatives, pesticides, and personal care products (Paterni et al. 2017). Although most XEs represent a risk to human and wildlife health, not all XEs have been shown to be harmful. Because most phytoestrogens are ER ligands with more affinity for ERβ over ERα (Paterni et al. 2014), preventive and therapeutic applications of these compounds are profusely studied for many conditions where ERα plays a key role such as osteoporosis (Słupski et al. 2021), chronic degenerative diseases (Bernatoniene et al. 2021), menopausal symptoms (Abdi et al. 2021), endometriosis (Bartiromo et al. 2021; Cai et al. 2021), cardiovascular disorders (Delgado et al. 2020; Silva 2021), and different types of cancer (Wang et al. 2021). XE types, sources, and health effects are summarized in Fig. 2.

Many XE are agonists of ER although they could also interact with the receptor or the co-regulators involved in E2 signalling pathways in ways different from those from endogenous hormones (Hall et al. 2002; Li et al. 2018). This diversity in the mechanism of action includes upregulation of ER expression, thereby increasing response or sensitivity to endogenous E2 as well as XEs, and the production of endogenous E2 (Holloway et al. 2008; Vandenberg et al. 2020). A chart comparing E2 and XEs actions is provided in Fig. 3.

Current Screening Methods

As mentioned above, human exposure to XEs causes significant health disorders. Since XEs are widely spread in the environment, humans can be exposed to them (occupational or accidental exposure) by inhalation, ingestion, or dermal contact (Kabir et al. 2015). Like E2, XEs usually exert effects at low doses (Pino et al. 2019). Although analytical methods are important to identify and quantify the XEs present in biological/environmental samples, they fail to predict the biological effects caused by them. Therefore, current studies focus mainly on detecting biological effects rather than the identity of the XE itself. The EPA's Endocrine Disruption Screening and Testing Advisory Committee (EDSTAC) proposes a battery of 11 assays to evaluate chemicals for potential endocrine effects by combining five in vitro and six in vivo assays (EDSTAC 1998). In this section, we briefly describe in vitro and in vivo screening models and tests commonly used to analyze the effects of EDCs,

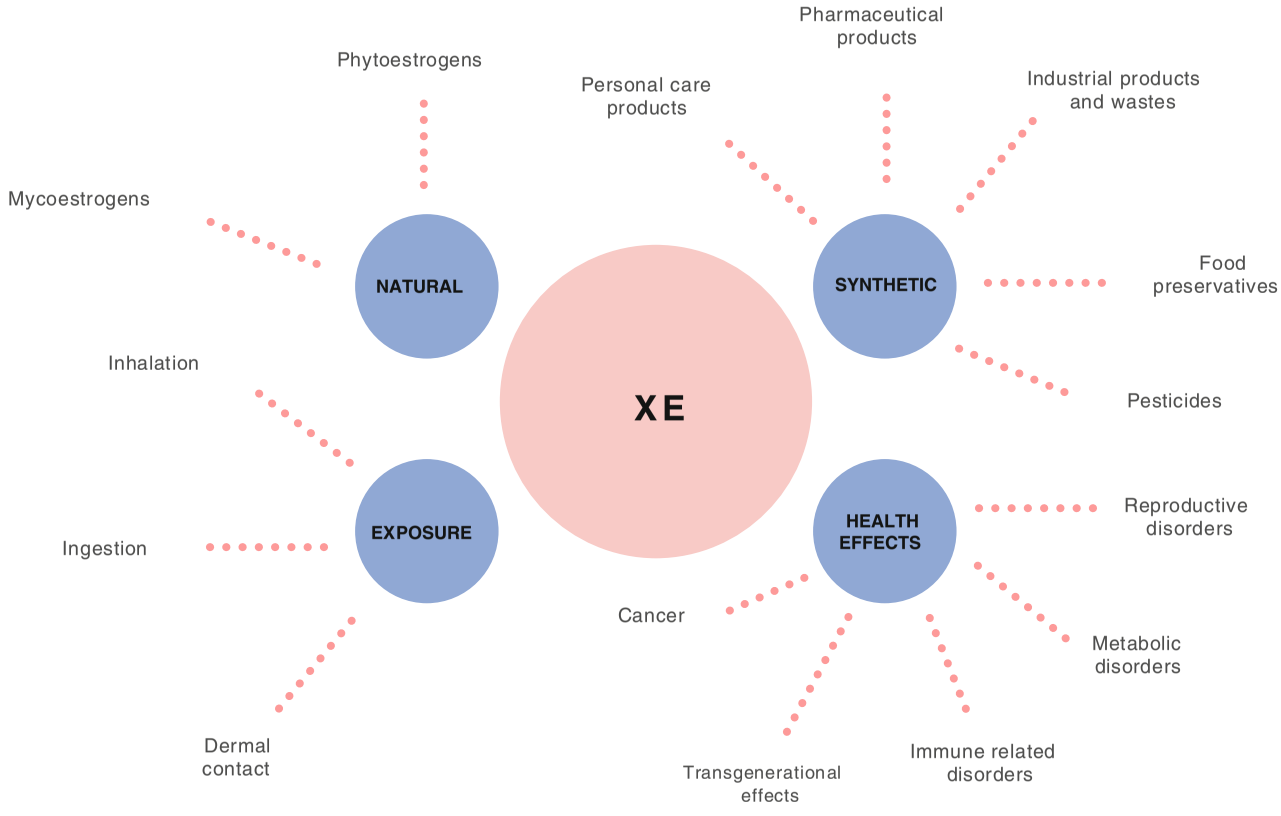

Fig. 2 Xenoestrogen overview. Xenoestrogens (XE) can be classified into two groups: natural and synthetic compounds. The first group is comprised of mycoestrogens (e.g., zearalenone) and phytoestrogens (e.g., isoflavones). Synthetic compounds, such as parabens, are present in personal care products. DES

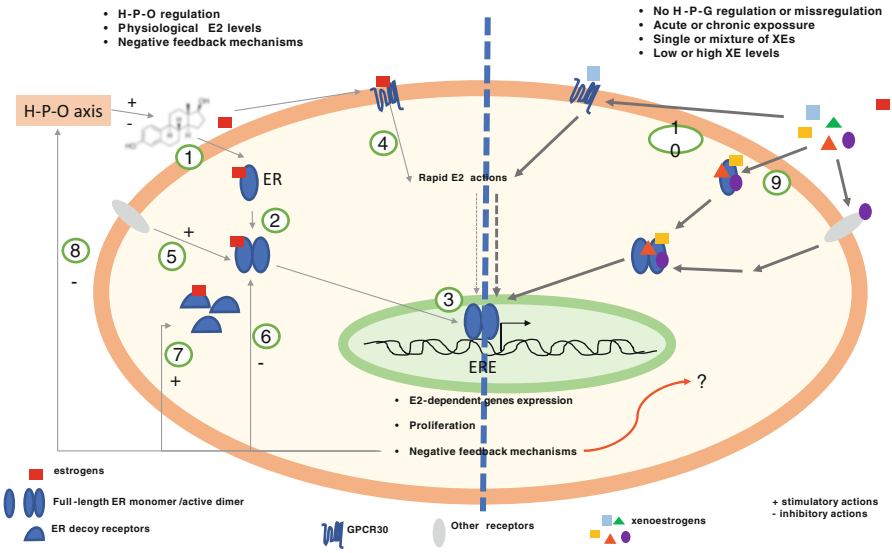

Fig. 3 Comparison between effects of estrogens and xenoestrogens. Estrogens (E2) produced by ovaries (1) can act in hormone-responsive tissues through their E2 receptors (ER) of cytoplasmic localization. Upon binding, ER activates and rapidly dimerizes (2) then acting as a transcription factor by binding E2-response elements (EREs) located in the promoter regions of E2-targeted genes (3), many of them directly related with cell proliferation. E2 also bind ER located in plasma membrane (GPR30) which triggers E2 rapid actions (4) characterized by activation of different signalling pathways that can also affect E2-dependent gene expression. Under certain conditions, E2 signalling pathway can also be cross-activated by other receptors, such as IGF1-R (5). Depending on E2 levels and the tissue involved, negative regulatory feedback mechanisms are simultaneously activated, including downregulation of ER (6), expression of truncated (decoy) ER isoforms (7), and inhibition of E2 release by negative feedback at hypothalamic, pituitary, and ovary levels (H-P-O axis) (8). Exposure to one XE or a mixture is frequently associated with E2-like unregulated effects. XEs can act alongside or instead of endogenous E2. They can display some or all of E2's known effects by acting alone or synergistically on ER (9) and/or GPCR30 (10), promoting their activation or hyper-activation, or on E2 signalling pathway (11). Many XEs compete with E2 for binding ER and some bind ER with more affinity than E2 itself, thus exerting strong or long-lasting responses (12). Acute or chronic exposure to XEs may have very diverse consequences on endocrine pathways, all characterized by disruption of endocrine regulation at multiple points

Fig. 2 (continued) and EE2 are two examples of pharmaceutical products. BPA is widely used in plastic industries, butylated hydroxyanisole is used as food preservative, and chlorpyrifos is broadly used as a pesticide. Humans can be exposed to xenoestrogenic compounds by inhalation, ingestion, or dermal contact. Acute or chronic exposure adversely affects health: reproductive disorders such as infertility, metabolic disorders since some are considered obesogens, immune-related disorders due to the important role of estrogen pathway in immune system. Transgenerational effects were also found after maternal exposure. Finally, several types of cancer are consequence of xenoestrogen exposure (Paterni et al. 2017)

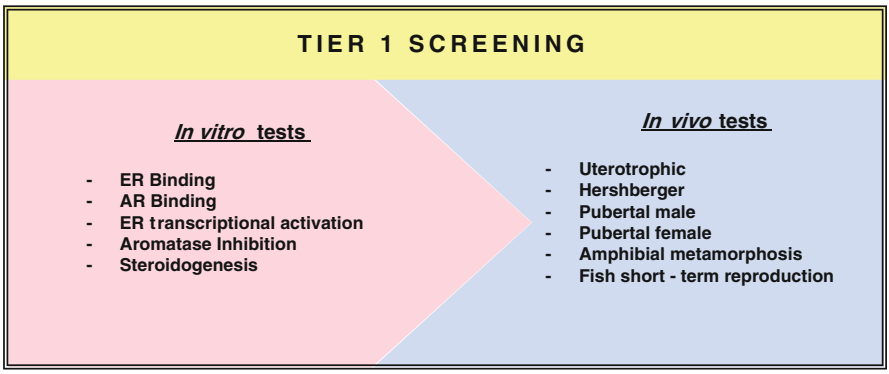

Fig. 4 Scheme of US EPA' Endocrine Disruptor Screening Program battery. This battery includes 11 Tier 1 screening assays divided into in vitro and in vivo tests designed to evaluate EDC activity. Bold written assays are described in "Current Screening Methods" section. ER: estrogen receptor. AR: Androgen receptor. For more details about methods and protocols, refer to EDSP test guidelines (https://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-890-endocrine-disruptor-screening-program)

focusing on those designed to test estrogenic activity in order to discuss the strengths and weaknesses of each method (Fig. 4).

Estrogen Receptor Binding Assay Using Rat Uterine Cytosol (ER-RUC)

Estrogen Receptor Binding Assay evaluates the ER binding capability of a chemical compound, providing direct evidence of its estrogenic potential. Ligand-binding assays were initially performed using radioactive ligands, but more recently, these assays are performed using nonradioactive ligands. Briefly, rat uterine cytosol containing ER is incubated in presence of labeled E2 and increasing concentrations of compounds being studied. If the compound binds ER, a displacement of labeled E2 occurs and therefore, the label intensity detected decreases (U.S. EPAa 2009). However, although the ligand-binding assay detects ER binding and it is suitable to test compounds that bind ER, it cannot distinguish between agonists and antagonists (Gea et al. 2020). Also, this test cannot provide information about downstream effects or characterize precisely the strength of the binding.

Estrogen Receptor Transcriptional Activation

This test is based on the ability of a chemical to form a complex with ER and to promote the activation of a gene reporter such as firefly luciferase. Endocrine Disruptor Screening Program Test Guidelines recommend Human Cell Line hER-α-HeLa-9903 to perform the assay since these particular cells stably express the

following construct inserts: hERα expression construct (encoding the full-length human receptor α) and a firefly luciferase reporter sequence accompanied by five tandem repeats of a vitellogenin E2-Responsive Element (ERE) under control of a mouse metallothionein (MT) promoter TATA element. If the chemical tested activates transcription through hERα, chemiluminescence is detected after adding a commercial luciferase assay reagent (U.S. EPAb 2009). An advantage is that this in vitro test can reveal whether a chemical behaves as an agonist of hERα. Also, a full-length human receptor β (hERβ) can be expressed separately to identify agonists for that receptor (Gea et al. 2020). However, HeLa-9903 cells are not easily available.

Steroidogenesis (Human Cell Line: H295R)

The aim of this assay is to determine whether a substance could affect E2 and testosterone (T) production in human adrenocarcinoma cell line H295R. Since it expresses all the key enzymes for steroidogenesis, this cell line is broadly accepted as a model to study E2 and T production (Gazdar et al. 1990; Rainey et al. 1993; Sanderson et al. 2002; Hilscherova et al. 2004; Hecker et al. 2006; Breen et al. 2010; Higley et al. 2010). Steroidogenesis assay is generally performed under standard cell culture conditions using a phenol red free media supplemented with a defined serum (Nu-Serum), previously determined for steroids. After incubation of H295R cells with the chemical being studied, culture media containing secreted steroids is collected for further E2 and T quantification followed by a viability test. Its advantages are that steroidogenesis test does not require animals, can be performed in few days, and provides information about hormone levels and cytotoxicity induced by the study chemical (U.S. EPAc 2009). However, culture media requirements are quite expensive and not easily available. Another drawback is that cell passage numbers must be strictly controlled since estradiol producing capacities of H295R cells with changed with passage/age (Hecker et al. 2006).

Rat Uterotrophic Assay

The Uterotrophic Assay is one of the first standardized tests to evaluate compounds with potential estrogenic effect (Bulbring and Burn 1935; Dorfman et al. 1936). It is a short-term in vivo assay in which uterine weight is measured after drug exposure (Reel et al. 1996). If the chemical studied acts as an agonist of natural E2, the rat uterus will respond by increasing weight as a consequence of water imbibition as an early step and then due to tissue growth (Jones and Edgren 1973). Currently two methods to test uterotrophic effects are available: the ovariectomized adult female method (OVX-adult method) and the immature non-ovariectomized method (immature method). The OVX model is preferred to test EDCs because it is more specific since immature rat has an intact

hypothalamic-pituitary-gonadal (HPG) axis, and therefore drugs can also act through this axis and not only through E2 receptors. The uterotrophic assay provides biological information that complements the ligand-receptor in vitro assay since the former shows the effect on E2 responding tissue in vivo whereas the latter cannot predict effects because it shows whether a chemical can or cannot bind to a receptor (U.S. EPAd 2009). Also, working with laboratory animals requires local ethical permits, protocol approvals, and professionals dedicated to guarantee animal welfare conditions. Further, the route of chemical administration, tissue distribution, clearance, and other metabolic parameters must be previously determined in order to obtain a reliable result from the uterotrophic test.

Rat Female Pubertal Assay

Female pubertal assay is based on the ability of a chemical to interact with endocrine system by promoting effects on pubertal development of intact juvenile female rats. This assay investigates whether a chemical elicits estrogenic or antiestrogenic effects (including those affecting receptor binding or steroidogenesis) as well as antithyroid effects which alter pubertal rat development through changes in hormone levels (luteinizing hormone, follicle stimulating hormone, prolactin, or growth hormone) or via hypothalamic function alterations. This assay requires 15 female pups for study including controls, and therefore animal availability is a limitation of this test. Also, before starting the assay, pups must be weaned on postnatal day 21 (PND 21) and all the litters ranked by weight. Treatments are administered daily by oral gavage from PND 22 through PND 42 and vaginal opening, weight gain and clinical signs are monitored and recorded daily. After vaginal opening, estrous cycle must be observed and recorded daily. Finally, on PND 42 animals are killed, truncal blood is collected for hormone detection and uterus, thyroid, ovary, and kidneys are evaluated for pathologic abnormalities and potential treatment-related effects (U.S. EPAe 2009). Female pubertal assay provides much information that must be carefully and exhaustively analyzed, making it a laborious process.

Fish Short-Term Reproduction Assay with Fathead Minnow (*Pimephales promelas*)

This in vivo test is based on the ability of a chemical to interact with fish endocrine system. In this screening assay, sexually mature male and female fish are exposed together to the study chemical for 21 days (U.S. EPAf 2009). During the test, survival, egg production, and fertility are recorded daily. After treatment, vitellogenin (VTG) and sexual characteristic of the fish are measured. Gonads are also analyzed to assess endocrine effects. Other optional endpoints are also assessable: gonadal somatic index (GSI) and plasma hormone levels (Wheeler et al. 2019). This assay provides not only information about sexual behavior and reproductive

features but also a biomarker for estrogenicity: VTG. VTG is a protein normally found in female fish which transports protein and lipid from the liver through the blood to the growing oocytes. Since it is expressed in response to E2 in females as well as in male fish, it is considered a biomarker of exposure to estrogenic compounds.

Soluble Guanylyl Cyclase

Nitric oxide-sensitive or soluble guanylyl cyclase (sGC; EC 4.6.1.2) is the main nitric oxide (NO) receptor. Upon binding NO, sGC activity increases more than 200-fold and catalyzes the formation of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP) which in turn acts as a second messenger and triggers several downstream signalling pathways, including activation of cGMP-dependent protein kinases (PKG), cGMP-gated channels, and cGMP-regulated phosphodiesterases (PDEs) (Derbyshire and Marletta 2012).

sGC is a heme-containing enzyme localizing in cytoplasm and is ubiquitously present in all eukaryote organisms and in almost every human cell type (Budworth et al. 1999; Schaap 2005). sGC is comprised of two subunits, α1 (GUCY1A3) and β1 (GUCY1B3), which form the most abundant and active enzymes (Montfort et al. 2017), although α2 and β2 subunits and several other alternative splicing variants have also been identified, all of them exhibiting less enzymatic activity than α1:β1 heterodimer (Budworth et al. 1999; Koglin et al. 2001). As sGC is an obligated heterodimer requiring a strict 1:1 α:β stoichiometry, relative abundance of α1 and β1 subunits as well as the presence of other isoforms or splicing variants establish another regulation point for sGC enzymatic activity (Sharina et al. 2011; Martin and Golunski 2014).

Both isoforms are encoded by separate genes; therefore, they can be regulated independently. Subunit expression and activity of sGC are modulated at multiple points from transcription to protein stabilization and half-life. It has been shown that sGC is affected at the expression level, alternative splicing and mRNA stability, activity, association with different proteins, trafficking and membrane translocation, and proteasome degradation, which underlines sGC as a key modulation factor of the nitric oxide pathway but also other pathways (Derbyshire and Marletta 2012).

sGC enzymatic activity as the main NO receptor plays a key role in the regulation of several physiological functions including neurotransmission (Wright 2019), vasodilation, platelet aggregation (Wobst et al. 2018), and metabolic processes (Litvinova et al. 2015), among others. Since NO pathway is central to the pathogenesis and progression of many conditions from airway, cardiovascular (Levine et al. 2012), and neurodegenerative diseases to cancer (McGinity et al. 2021), it is intensively studied as an attractive target for the development of drugs and therapeutic approaches.

Estrogen and Soluble Guanylyl Cyclase

Previous work from our laboratory demonstrated that both sGC subunits can be not only independently but also differentially regulated by E2 (Cabilla et al. 2006, 2009, 2011), even when they lack EREs in their promoter regions (Marro et al. 2008). In particular, we demonstrated that sGCα1 mRNA and protein expression increases after E2 acute or chronic treatment in anterior pituitary glands from female rats under several physiological conditions and also in in vitro experiments. We found that sGCα1 protein levels vary depending on physiological changes in E2 circulating levels during the estrous cycle (Cabilla et al. 2009). Moreover, although the sGCα1 gene promoter sequence lacks E2 consensus sites (Marro et al. 2008), we demonstrated that E2 increases both sGCα1 de novo transcription and translation of certain factors regulating sGCα1 and sGCβ1 protein levels (Cabilla et al. 2011). In all cases, we observed that E2 chronic or acute treatment did not alter or even lower sGCβ1 mRNA or protein expression (Cabilla et al. 2006, 2009, 2011). Subsequently, we also showed that sGCα1 protein levels increased in rats after chronic treatment with cadmium and arsenic – two well-known XEs – at low doses in uterus and pituitary gland (Ronchetti et al. 2016). This strong dependence between E2 or XE levels and sGCα1 expression provided the rationale to investigate whether sGCα1 could be a suitable exposure marker for screening XE activity of EDCs compounds.

sGCα1 Subunit as a Biomarker of Exposure

In vitro assays using standard culture methods are a quick, accessible, and relatively inexpensive way to investigate the putative E2-like activity of a compound. They represent an excellent first approach in toxicity screening tests, which are always followed by in vivo determinations for complete characterization of EDCs' biological effects. Several exposure markers have been chosen as biomarkers of exposition, mainly based on their sensitivity to E2 or E2-like compounds. However, some are not ubiquitous, exhibit low expression levels, or strongly depend on temporal developmental stages, making their routine use difficult.

Since sGCα1 is an abundant protein present throughout the zoological scale, as well as in several cell lines included in current screening tests, sGCα1 protein determination by Western blot has shown it is a reliable way to study the response to E2 or XE compounds. It was shown to be a suitable biomarker of exposition to XEs because of its high sensitivity to E2 and to a broad range of XEs at environmentally relevant doses. Remarkably, it also responds to some compounds that can direct or indirectly lead to E2 signalling activation without binding ER, thereby broadening the landscape of characterization of E2-like compounds. As sGCα1 gene lacks ERE in its promoter sequence, its expression levels can increase in response to ER coactivator recruitment or to ER corepressor inhibition, in any case leading to the same final response. All these findings are depicted in Fig. 5. Also, evidence showed that sGCα1 protein levels

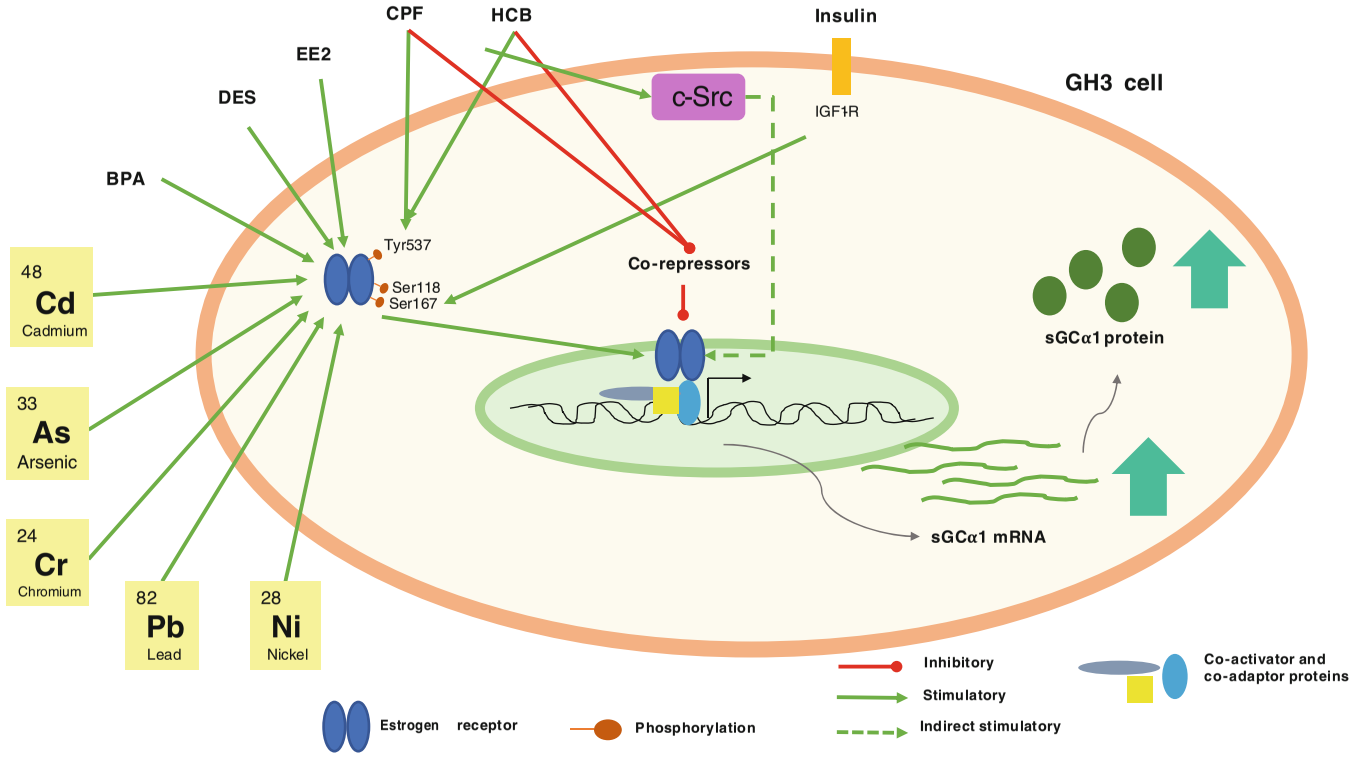

Fig. 5 Summary of XE actions on sGCα1 subunit expression. Using lactosomatotroph-derived GH3 cells, we assayed the response of sGCα1 subunit to XEs varying in structure and mechanisms of actions at relevant, low doses. Metals such as cadmium, chromium, lead, and nickel and the metalloid As bind and activate ER, as well as synthetic E2 derivatives 17α-ethynylestradiol (EE2) and diethylstilbestrol (DES), all having equal or more affinity than E2. All were tested in subnanomolar-picomolar concentration range. Bisphenol A (BPA) is known to bind ER with low affinity. We tested sGCα1 response to BPA in

are upregulated in response to androgens through the androgen receptor in prostate cancer cell lines (Cai et al. 2007). In this way, the hypothesis highlighting sGCα1 as a possible marker of exposure to EDCs with androgenic activity needs to be addressed.

Since sGCα1 determination is an in vitro assay, the use of animals in a specific maturation stage or with certain hormonal status is not required. Moreover, interference introduced by hormonal regulation or other hormone actions is avoided. Although in vivo studies are needed because they provide quality evidence of complex interactions in a complete system, the primary characterization of a potential XE in in vitro systems through highly specific biomarkers optimizes these studies, helping to reduce animal use. Importantly, unlike other biomarkers, E2- and XE-driven sGCα1 were shown to increase, positively correlating with proliferation in many cell lines and tissues from in vivo studies; therefore, sGCα1 as a biomarker is also associated with a measurable biological effect.

In sum, sGCα1 expression-based assay is a useful tool to be added to current in vitro estrogenicity tests to fully understand the behavior of known and emerging EDCs and their potential impact on wildlife and human health.

Applications to Other Diseases or Conditions

Besides showing several characteristics that make it a suitable marker of exposure to XEs, increasing evidence demonstrates that sGCα1 could also become an important factor in cancer biology. It has been shown that sGCα1 promoted survival, growth, and migration of androgen-dependent prostate cancer cells (Cai et al. 2007, 2012; Zhou et al. 2017). Previously, we showed the strong dependence of sGCα1 on E2 levels and E2-driven proliferation in hormone-dependent tissues and cell lines (Cabilla et al. 2006, 2009). We have recently shown that sGCα1 displays protumoral actions in endometrium (ER-dependent) and cervical (ER independent) tumor cells (Ronchetti et al. 2019). Furthermore, some studies have revealed that sGCα1 expression levels positively correlated with tumor grade together with other classic breast cancer markers as ER and progesterone receptor in biopsies from patients (Mohammadoo-Khorasani et al. 2019, 2020). sGCα1 expression was shown to be higher in breast cancer compared to normal breast tissue. Moreover, sGCα1 expression seems to be moderate to high in almost all tumor types, with very few exceptions (The Human Protein Atlas; Uhlen et al. 2017). Altogether, this growing evidence supports the role of sGCα1 as a protumoral factor and potentially as a prognostic and/or therapeutic target which needs to be intensively investigated.

Fig. 5 (continued) nanomolar concentrations. Chlorpyrifos (CPF) and hexachlorobenzene (HCB) do not bind but activate ER through different mechanisms. They promote phosphorylation at ER tyrosine residues, downregulate ER corepressors and activate c-Src pathway, which can indirectly activate ER transcriptional activity. Other ligands not binding ER, such as insulin at high doses (submicromolar concentrations), can activate ER signalling pathway by cross-talk mechanisms involving phosphorylation of ER at serine residues. All these compounds at low or very low relevant doses are able to increase sGCα1 protein levels detected by Western blot (Pino et al. 2019)

Mini-dictionary of Terms

- **Endocrine-disrupting chemicals**. Compounds that interact with endocrine system by mimicking, blocking, or interfering with the action of endogenous hormones.
- **Estrogen**. The main female hormone involved in many physiological and physiopathological processes, such as reproductive development, regulation of menstrual cycle, many breast cancers, and autoimmune diseases, among others.
- **Marker of exposure**. An exogenous compound or its metabolite or an endogenous biomolecule affected after exposure to a xenobiotic measured in an organism.
- **Soluble guanylyl cyclase α1 subunit**. One of sGC's subunit that is upregulated by E2 and various XEs.
- **Soluble guanylyl cyclase**. A heterodimeric enzyme composed of α and β subunits involved in nitric oxide signalling pathway with ubiquitous expression.
- **Xenoestrogen**. A natural or synthetic compound able to behave as endogenous estrogen.

Key Facts of Endocrine-Disrupting Chemicals

- Endocrine disruptor chemicals are synthetic or natural compounds that interfere with hormone action.
- They comprise a wide variety of compounds from organic molecules to heavy metals; many are environmentally persistent with high bioaccumulation capacity.
- Xenoestrogens can mimic estrogen actions which may lead to a number of endocrine disorders and diseases including cancer.
- Some xenoestrogens are even more potent than estrogen itself and display actions at lower doses.
- The lists of EDCs/xenoestrogens are constantly growing as new compounds or their derivatives are incessantly produced.
- Toxicity assays to characterize EDCs include a first battery of in vitro determinations by assessing estrogenic activity followed by in vivo tests.

Summary Points

- Endocrine disrupting chemicals (EDCs) represent a threat to human and wildlife health.
- Xenoestrogens (XE) are EDCs capable of mimicking, impeding or interfering with normal endogenous estrogen actions.
- Current methods for screening XE activity include in vitro and in vivo assays.
- In vitro testing of XE is a low cost, rapid, and efficient method to quickly identify EDCs with E2-like activity.

- Most classical markers of XE exposure present some disadvantages such as low or temporarily low expression or tissue-specificity.
- Soluble guanylyl cyclase α1 (sGCα1) subunit is relatively abundant and ubiquitously expressed in all tissues and many cell lines.
- sGCα1 is highly sensitive to E2 levels and responds to a wide range of XEs varying in structure and mechanisms of action.
- sGCα1 is an effective marker of exposure to EDCs since it is sensitive to very low doses of XEs and compounds not binding ER.

Cross-References

► Pro-inflammatory Markers of Environmental Toxicants

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Serum Paraoxonase 1 as a Biomarker in Toxicology

2

Başak Gökçe

Contents

Abstract

Biomarkers are used in many areas of health to diagnose the presence of disease, monitor disease progression, track drug distribution in the body, or determine exposure to chemicals. The increase of free radicals damages biochemical macromolecules and increases the formation of new toxic compounds such as enhanced oxidation protein products. Organophosphate (OP) compound exposure, oxidative stress, and lipid peroxide have been associated with DNA damage, various types of cancer, aging, degenerative disease, and inflammatory diseases in humans. For this section, the evaluation of paraoxonase (PON1), a liver enzyme that can hydrolyze toxic OP compounds, is bound to HDL (High-density lipoprotein) in serum and draws attention with its strong antioxidant properties, as a biomarker is discussed. In this chapter, PON1 as a toxicological biomarker was reviewed in three cases: organophosphate exposure, lipid peroxidation, and liver toxicity. PON1 status against toxic damage; both serum PON1 level and PON1 polymorphisms were discussed together in the light of studies. Finding new reliable biomarkers is an area of research that has become increasingly important in recent years.

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Keywords

Paraoxonase 1 · Biomarker · Organophosphate · Liver · Oxidative stress · High-density lipoprotein · Toxic · Disease · DNA damage

Abbreviations

AChE	Acetylcholinesterase
BuChE	Butyrylcholinesterase
CB	N-Methyl-carbamate
CHD	Coronary heart disease
ChE	Cholinesterase
CLD	Chronic liver disease
DZO	Diazoxon
HDL	High-density lipoprotein
His-His	Histidine-histidine
HTL	Homocysteine thiolactone
Km	Michaelis constant
LDL	Low-density lipoprotein
LDLs	Low-density lipoproteins
OP	Organophosphate
PCR	Polymerase chain reaction
PON1	Paraoxonase 1
Vmax	Maximum velocity

Introduction

Biochemical Properties and Gene Structure of Paraoxonase 1

In the past few years, there have been rapid advances in the field of paraoxonase research. Paraoxonase (PON1) is a Ca^{2+}-dependent enzyme and associated with HDL in serum; it is capable of hydrolyzing many substrates, including highly toxic nerve agents and various organophosphorus insecticides, oxidized lipids, and a number of drugs (Fig. 1). The paraoxonase 1 enzyme is classified by the Enzyme Commission of the International Union of Biochemistry and Molecular Biology as an aryldialkylphosphatase (EC 3.1.8.1). Paraoxonase 1 enzyme has organophosphate activity (e.g., substrate, paraoxon, diazoxon), lactonase activity (e.g., dihydrocoumarin, homocysteine thiolactone), and arylesterase activity (e.g., substrate phenyl acetate) (Fig. 2).

The catalytic mechanism of the paraoxonase enzyme has been elucidated. The Ca^{+2} ion, which has catalytic activity, is 2.2A away from the negatively charged oxygen of the phosphate ion in the substrate. The His pair in the active site takes a proton of the water molecule, increasing its nucleophilic strength. The hydroxyl ion formed attacks the carbonyl or phosphate ester. The complex formed at this time is

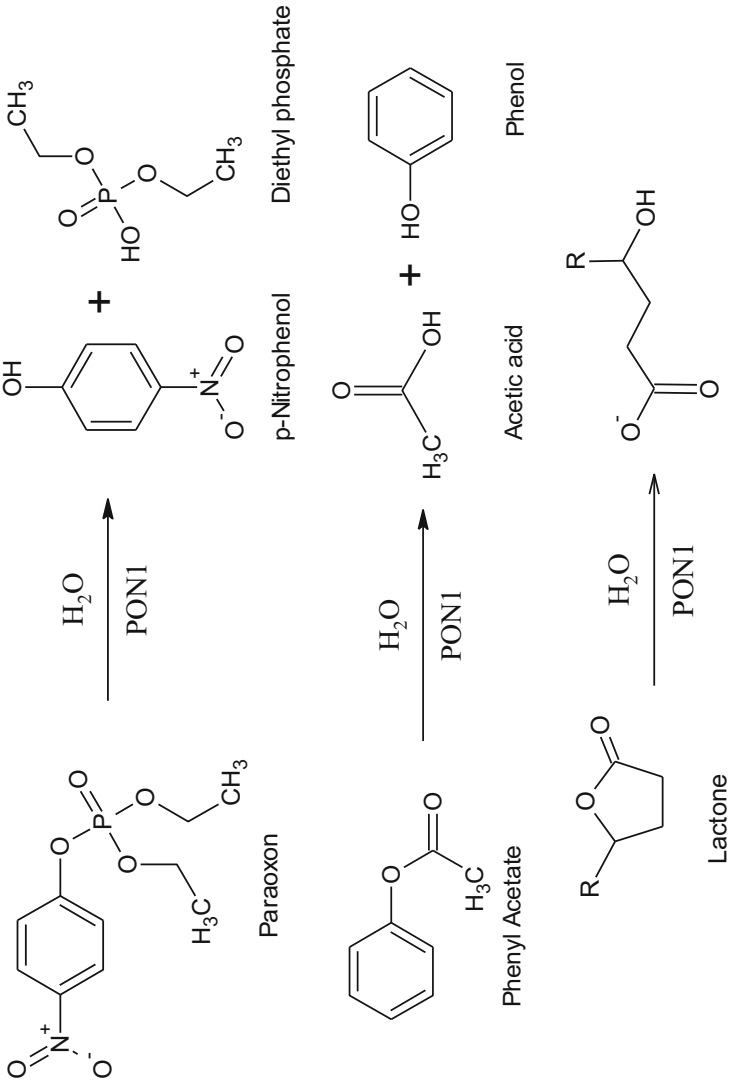


Fig. 1 Activities of paraoxonase 1

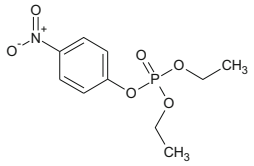
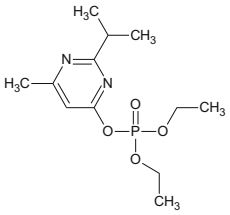
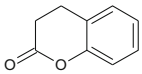
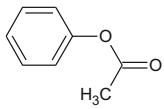
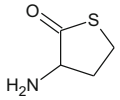
Organophosphates activity		Lactonase activity	Arylesterase activity
 Paraoxon	 Diazoxon	 Dihydroqoumarin	 phenil acetat
		 Homo cysteine thiolactone	

Fig. 2 The structures of paraoxonase 1 substrates

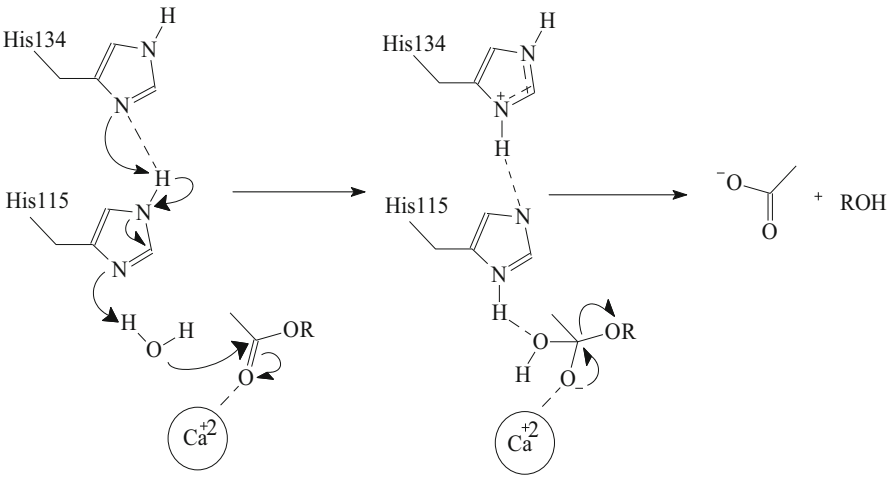


Fig. 3 The catalytic mechanism of paraoxonase 1

stabilized by Ca^{+2} ion as a result of a tetrahedral plane. The Ca^{+2} ion in the formed structure moves away from the negatively charged oxygen, and the bond breaks down on the phosphate or carbonyl again, and the ester bond is broken (Fig. 3).

PON1 is mainly synthesized by the liver, but histochemical experiments have shown the expression of PON1 in many different mammalian tissues, not only in the liver in humans. Since it is dependent on HDL, it is associated with many diseases. PON1 level has been shown to be important in protecting against atherosclerosis, possibly through PON1's physiological role in preventing lipid peroxidation (James 2006). Low serum PON1 levels are associated with many diseases, such as various liver diseases, kidney failure, diabetes, rheumatoid arthritis, and atherosclerosis

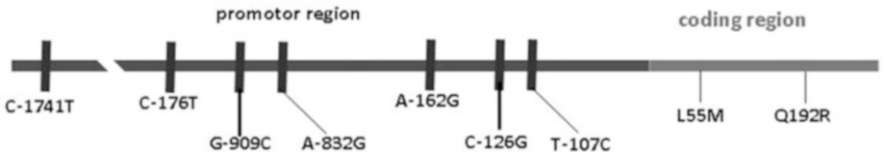


Fig. 4 Polymorphisms in the promoter and coding regions of paraoxonase (PON) 1. (Adapted from Camps et al. (2017))

(Goswami et al. 2009). These metabolic diseases are characterized by low PON1 activity due to dysfunctional HDL (Navab et al. 2007). Now, it is well-known that PON protein plays a vital role in toxicology, inflammation, and infection (Schrader and Rimbach 2011). Although the effects of lifestyle, pharmaceutical modulators, and diet on PON1 activity are known (Costa et al. 2005), the difference in PON1 levels between individuals has the greatest effect due to genetic polymorphisms of PON (Deakin et al. 2002).

The polymorphism of the coding region of the paraoxonase 1 enzyme is characterized by the methionine to leucine polymorphism at position 55 (PON1 Leu55Met or PON1 L55M) and the arginine to glutamine polymorphism at position 192 (PON1 Gln192Arg or PON1 Q192R). PON1 Q192R correlates with the QQ, QR, and RR genotypes, whose polymorphism corresponds to the AA, AB, and BB phenotypes, respectively (Fig. 4). Also, PON1 Q192R polymorphism shows the capacity of PON1 to inhibit LDL oxidation. The Q isoform is more efficient than the R isoform (Mackness et al. 1997; Aviram et al. 1998). The PON1 Q192R polymorphism differs on activity depending on the substrate. For example, paraoxone is hydrolyzed faster by the R isoform, while diazoxon is hydrolyzed faster by the Q isoform. PON1 promoter region T-108C and the PON1 coding region L55M polymorphisms are associated with different serum PON1 concentration levels and different activities too. PON1-55L allele shows higher PON1 serum protein and mRNA levels, so it's higher activity than the PON1-55M allele. PON1-108C allele has larger promoter activity and different serum activity than PON1-108T allele. Many other polymorphisms affect the serum PON1 level less.

Applications to Prognosis, Other Diseases, or Conditions

Organophosphate Exposure

Based on recent studies, biomarkers evaluating human exposure to toxic compounds and their relationship to PON1 will be discussed in this section. Organophosphate (OP) chemicals produced to control insects and pests are unfortunately ubiquitous in the environment. In the last half century, the unconscious use of N-methyl-carbamate (CB) and organophosphate (OP) insecticides has increased considerably around the world. They are especially used in some developing countries of the world to combat a lot of unwanted insects and pests. Paraoxonase (PON1) enzyme metabolizes highly toxic oxon forms of some OPs and pesticides which are commonly used. Some

individuals' health may be more sensitive to harmful compounds. This situation is based on studies with experimental animals; it is thought that paraoxonase (PON1) levels of individuals are an important determinant of susceptibility to insecticides. Studies with transgenic mice have indicated that low serum PON1 activity level is associated with brain acetylcholinesterase inhibition as measured by exposure to diase oxon and chlorpyrifos oxon (Li et al. 2000). Moreover, not only the paraoxonase concentration in the serum but also the Q/R polymorphism at the PON1 192 position affects an individual's OP sensitivity. An individual's PON1 level and polymorphism may be an important determinant of susceptibility to these chemicals. A study of acute OP intoxication observed that cases had significantly higher PON1 Q192 genotype and lower paraoxonase activity than controls (Davies et al. 1996). The PON1 polymorphism differentially impacts the activity of the catalysis of some PON1 substrates. Both PON1 plasma level and PON192 genotype should be evaluated together in determining the risk of exposure of individuals to disease and toxic compounds (Table 1). It was evaluated the association of OP exposure and PON1 Q192R genotype among workers in fruit-growing regions in South Africa. QQ homozygotes or QR heterozygotes were almost three times more likely than RR individuals to show more than one symptom of chronic OP toxicity (Li et al. 2000).

A study suggested that purified serum PON1 protects against OP exposure in mice and using PON1 as a catalytic converter for the treatment of hazardous compound exposures (Li et al. 2000). The difficulties arising from the purification of a quite hydrophobic protein PON1 have also been shown to be an obstacle to the development of the catalytic scavenger (Gan et al. 1991). In general, most PON1 purification attempts have studied human blood for serum as the enzyme material, where the protein is most abundant, and purification has been accomplished by several chromatographic methods (Furlong et al. 1991). In recent developments, the number of steps required to purify human serum PON1 has been decreased (Gencer and Arslan 2009). Evidence has been obtained from different studies that the use of recombinant human PON1 would be appropriate for therapeutic protection against OP exposure. As a result of these observations, it has been shown that human PON1 can be produced with sufficient purity by biotechnological way from bacterial cells and can be injected without any negative effects.

Cholinesterase (ChE) enzymes have two types found in the blood; the first, acetylcholinesterase (AChE), is related with red blood cell membranes, while the

Table 1 Catalytic effect of different OP substrates on purified human serum $PON1_{192}$. (Adapted from Li et al. (2000))

	Paraoxon hydrolysis		Diazoxon hydrolysis		Chlorpyrifos oxon hydrolysis	
	$PON1_{Q192}$	$PON1_{R192}$	$PON1_{Q192}$	$PON1_{R192}$	$PON1_{Q192}$	$PON1_{R192}$
Km (mM)	0.81	0.52	2.98	1.02	0.54	0.25
Vmax (U/mg)	0.57	3.26	222	79	82	64
Vmax/km	0.71	6.27	75	77	152	256

second, butyrylcholinesterase (BuChE), is found in serum. Inhibition of both AChE and BuChE is considered to be biomarker in determining the early biological effects associated with CB/OP exposure. In general, inhibition of AChE is thought to be a better biomarker for determining toxicity, while inhibition of BuChE is a more reliable indicator of most CB/OP exposures. Chlorpyrifos, diazinon, and malathion inhibit BuChE more effectively than AChE. By hydrolyzing the neurotransmitter acetylcholine, AChE has a vital role in the regulation of peripheral and central nervous system transmissions. The major effect of CB/OP toxicity is inhibition of neural acetylcholinesterase (AChE) enzyme activity. In one study, it was determined that PON1 is more ideal as a biomarker for assessing OP exposure, since pesticide handlers are more exposed to hazardous compounds, pesticides, than other agricultural workers (Li et al. 2000). For some OPs, such as chlorpyrifos, PON1-Q192R polymorphism is significant as it determines the catalytic mechanism of hydrolysis. Nonetheless, in the findings obtained, it was determined that PON1 activity showed great differences between individuals who have Q192R genotype. The serum PON1 activity level is meaningful in all wide variety of OPs metabolized by PON1 within the physiological ranges determined. Determination of both serum PON1 activity level and PON1 genotype provides a more meaningful of overall PON1 status rather than Q192R genotype only (Jarvik et al. 2003). Paraoxonase 1 activity level can be affected by nutritional status, smoking, and medications (Mackness and Mackness 2015). But most researches indicate that serum PON1 activity level tends to remain constant over time and is regulated by genetic factors, specifically the PON1 polymorphism promoter region C-108T (Davies et al. 1996). The functional PON1 Q192R genotype of the individual can be identified using the enzymatic assay method with two substrates, which are paraoxon and diazoxon. In this assay, activity is measured using these substrates in plasma samples. The basic principle of this method has been demonstrated in previous studies in the literature. In a 2003 study, the accuracy of the observed Q192R genotype experiment with two substrate analyses was demonstrated by the excellent agreement between the genotype predicted as a result of PCR experiments (Jarvik et al. 2003).

Animal studies related to PON1 polymorphism have shown that PON1's relationship in determining OP toxicity is more sensitive when both phenotype and genotype are considered together, rather than just genotyping. Findings from several epidemiological studies and extensive animal studies have clearly shown that interindividual variability of PON in susceptibility to OP exposure should be considered.

Lipid Peroxidation

Atherosclerosis is a chronic inflammatory disease involving both adaptive and innate immunity. As a result of peroxidation of fatty acid residues in triglycerides, phospholipids, and cholesterol esters, reactive aldehydes are formed, and these initiate inflammatory responses.

Lipid peroxidation is the attack of unsaturated fatty acids by free radicals. Due to the oxidation products formed during the process, it is associated with various diseases. High LDL concentration or oxidized LDLs are directly associated with

the risk of atherosclerosis development. Dysfunction of HDL may be associated with decreased vascular cholesterol flow rate, but the most common consequence is impaired anti-inflammatory/anti-oxidative function. Serum PON1 is found to be associated with high-density lipoprotein (HDL) particle that has a potential positive role in lipid metabolism (Mackness et al. 1991). Paraoxonase 1 has the ability to inhibit lipid peroxidation on low-density lipoproteins (LDLs) both in vitro and in vivo (Watson et al. 1995). HDL can prevent the progression of CHD by delaying the oxidation of LDL. The mechanism for how PON1 delays LDL oxidation has not been fully proven. Several proteins on HDL (Apo AI, apoM, GSPx, PLA2, LCAT), with PON1, contribute to this antioxidant property of HDL (Fig. 5). In studies performed in cell culture, PON1 has been shown to have different atheroprotective effects such as inhibiting cholesterol synthesis, increasing cholesterol efflux, and reducing macrophage oxidative stress. A possible association between PON1 and atherosclerosis showed that patients with myocardial infarction had lower serum PON1 activity than the control group. In patient control studies, low serum PON1 activity levels were seen as a biomarker associated with susceptibility to CHD development. In addition, there are different studies in the literature that PON1 activity is a risk factor for the development of atherosclerosis, regardless of HDL concentration level (Mackness et al. 2003). In some studies, the relationship between risk factors for CHD and PON1 polymorphism has been investigated. Findings, although PON1-Q192R polymorphisms are a risk factor for CHD, no link was found with other PON1 polymorphisms (Ferre et al. 2005).

Low serum PON1 activity levels are associated with many inflammatory diseases as a possible biomarker, including systemic lupus erythematosus, rheumatoid arthritis, and diabetes mellitus (Goswami et al. 2009). Also, these serious health problems are characterized by having dysfunctional HDL, which is hypothesized to be caused by low PON1 activity. Further studies are needed to determine whether low PON1 causes the development of these inflammatory diseases. However, low serum PON1

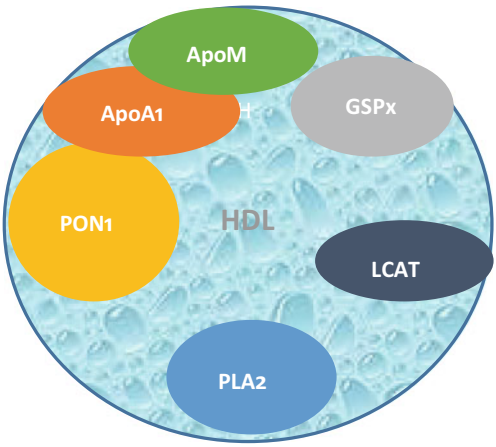


Fig. 5 Schematic representation of HLD with proteins on. Apo AI, apolipoprotein AI; apoM, apolipoprotein M; GSPx, glutathione peroxidase; PLA2, phospholipase A2; PON1, paraoxonase 1; LCAT, lecithin-cholesterol acyltransferase

activity levels, together with other tests, can be seen as a potential marker of inflammatory diseases.

A few studies have shown that there is a decrease in serum PON1 activity with advancing age and consequently a decrease in the protective functions of HDL. In addition, polymorphisms in the PON1 gene are related in healthy aging. Oxidative stress is a situation where the balance between oxidant formation and antioxidant defense is disrupted in the direction of oxidants. It is closely related to the pathogenesis of many pathological conditions and aging. In a study, the results demonstrate that cellular PON1 has a significant role in aging and this multifunctional protein may be associated with aging. Quality aging is an important target for improving living standards, so more studies are needed on the current function of PON1 (reviewed in Mackness et al. 2015).

Considering the studies, it is clear that PON1 contributes to the antioxidant characteristic of HDL and therefore PON1 has atheroprotective role. However, larger epidemiological studies are needed on all possible functions of PON1.

Liver Toxicity

The liver has a wide variety of functions such as synthesis of coagulation factors and other proteins, excretion of bile, and detoxification of harmful products of metabolism. Liver disorders are inflammatory conditions associated with increased plasma cytokine and chemokine concentrations (Visser and van der Heijde 2009). In chronic liver disease (CLD), which is characterized by severe worsening of liver function for at least 6 months, oxidative damage affects the pathological changes that cause liver cirrhosis and hepatocellular carcinoma and cirrhosis (McClain et al. 1999). Since the enzyme paraoxonase 1, which is produced in the liver and subsequently released into the serum with HDL, has a protective effect against oxidative damage, it is reasonable to find a relationship between liver failure and PON1. Studies first performed in the 1970s showed an important reduction in PON1 level among small patient groups with liver cirrhosis (Burlina and Galzigna, 1974). Later, these results were confirmed by examining a larger population of patients with chronic liver injury. When compared to the healthy control group, a significant reduction in serum PON1 activity level was found in patients with chronic hepatitis and a dramatic reduction in patients with liver cirrhosis. In the same study, Ferré et al. concluded that there is a genetic relationship between chronic hepatitis C infection and the PON1-192R allele polymorphism (Ferré et al. 2005). In another study, it was determined that the PON1-192Q allele has stronger effect on hydrolyzing lipid peroxides than the PON1-192R allele (Aviram et al. 2000). In chronic liver diseases, various hypotheses have been proposed as to why serum PON1 activity is reduced (Aviram et al. 1998). Patients suffer from liver damage if they have increased free radicals; this may inactivate PON1 by hydrolyzing lipid peroxides. Changes in HDL structure related to PON1 may effect PON1 activity. When PON1 synthesis is altered by the liver, PON1 activity may decrease accordingly. Changes in serum PON1 levels with alcohol intake depend on the degree of hepatic damage (Deakin et al. 2005).

Studies performed so far have shown that altered serum PON1 levels due to alcohol consumption are not a major factor in being affected by PON1 genetic

polymorphisms (Costa et al. 2005). In the study among chronic alcohol abusers, subjects were grouped according to their level of hepatic disease. The case studies performed have shown that the serum PON1 activity level is reduced in alcoholic individuals and the level of alcohol dependence is correlated with the extent of liver dysfunction. Also, in the same study, an inverse correlation was found between plasma malondialdehyde concentration and serum PON1 activity level in severe chronic alcoholic individuals (Marsillach et al. 2007).

Different nonphysiological substrates can be used to measure PON1 activity, as there is not yet a "natural" substrate for paraoxonase 1. Substrates, especially in the form of OP, are very toxic. The paraoxon substrate, after which it is named, has an LD50 of 0.5 ppm and is not healthy to measure for automated analytical assay systems. Using highly toxic pesticide-derived substrates to measure PON1 activity, which is one of the tests used in the determination of liver damage, is a great dilemma. Fortunately, new healthier method for serum PON1 activity measurement was developed, such as lactonase activity, using nontoxic substrate as homocysteine thiolactone (HTL), which makes it more practical to propose as a biomarker for liver toxicity (Marsillach et al. 2008).

Fatty liver disease in dairy-type cows is a very serious metabolic syndrome that affects most of the herd after calving. It is often associated with low immunity and fertility levels and can even lead to death from liver failure if left untreated (Katoh 2002). For the determination of fatty liver in dairy cows, serum PON1 testing will have an important clinical impact. General biochemical tests to detect liver dysfunction are not sufficient to diagnose fatty liver in dairy cows (Bobe et al. 2004). Therefore, over time, the definitive way to diagnose fatty liver has become the pathophysiological examination of the liver – biopsy. However, this is a costly procedure for dairy farms. Serum PON1 activity measurement to biochemical tests indicates that it will have greater impact in the dairy industry (Mackness et al. 1996).

In a study in dairy cows, serum PON1 activity (arylesterase, paraoxonase, and lactonase) and other hematological and biochemical parameters were measured in Holstein-Friesian cows with 46 fatty liver cases and 46 healthy cows as control group. It was found that serum PON1 activity levels were lower in dairy cows with fatty liver, affecting fertility and immune function, than in healthy cows (Bobe et al. 2004).

Determination of serum PON1 activity as a biomarker has been suggested to be useful in assessing the degree of liver failure. Measurement of serum PON1 level is a powerful diagnostic effect to distinguish patients with liver disease from healthy control subjects. When serum PON1 activity is added to known liver function tests, it will make more reliable contributions in increasing the general sensitivity (Ferre et al. 2005; Camps et al. 2007).

In addition, paraoxonase 1 is closely associated with the improvement of liver function. The value of serum PON1 activity and its effects in patients with severe liver damage and requiring transplant therapy were investigated (Xu et al. 2005). Serum PON1 activity levels were found low when hepatic arteries occluded in liver transplant patients, but it also tended to increase. This finding indicates that the fact

that PON1 activity is included in routine liver function tests will also be useful in determining whether liver transplantation is successful or not.

Key Facts of Cirrhosis

- It is the name given to severe damage to the liver.
- It is caused by many liver diseases such as hepatitis and chronic alcoholism.
- Cirrhosis slows the normal blood flow in the liver and thus increases the pressure in the vessel that brings the blood from the intestines and spleen to the liver.
- The liver damaged by cirrhosis may become unable to clear toxins. Accumulation of these toxins in the brain can cause confusion and difficulty concentrating.

Key Facts of Coronary Heart Disease (CHD)

- It is the name given to diseases caused by problems that block the vessels that feed the heart.
- Some plaques called "hardening of the arteries" can prevent the flow of the blood in the vessels.
- The most common symptom of the disease is chest pain.
- The most important complication of CHD is heart attack.

Mini-dictionary of Terms

Antioxidant: It is a compound that repairs damage at the cellular level caused by reactive oxygen radicals.

Apo-A1: It is one of the apo proteins found on HDL and interacts with cells to mediate cholesterol ester delivery.

Atheroprotective: It means that it protects against the formation of atherosclerosis.

HDL (high-density lipoprotein): It provides the transport of cholesterol from tissues and vessels to the liver.

Km: It is the unit that indicates the affinity of the enzyme for its substrate and equal to the substrate concentration required to reach half the maximum reaction rate.

Malondialdehyde: A specific or quantitative indicator of fatty acid oxidation correlates well with the degree of lipid peroxidation. It can be detected in the blood or urine.

Substrate: A molecule that binds to the active site of an enzyme and chemically converts it to a product.

Vmax: It is the reaction rate reached when the active site of the enzyme is completely saturated by the substrate.

Summary Points

So far, studies with experimental animals and case studies indicate the need for more reliable testing of the diagnosis of liver toxicity, lipid oxidation, and organophosphate exposure. Paraoxonase 1 (PON1) is a high-density lipoprotein (HDL)-associated serum enzyme that can catalyze a wide range of substrates. The reason why PON1 is discussed in this section as a possible toxicological biomarker in the future is because of the superior properties of this enzyme. Paraoxonase protects against exposure to some organophosphorus (OP) pesticides by hydrolyzing toxic metabolites such as oxon and sarin. Also, PON1 is effective in protecting against cardiovascular diseases by metabolizing oxidized lipids. Not only serum PON1 level but also PON1 polymorphism gives preventive and precautionary opinions about toxicity susceptibility of individuals. Some studies suggest that adding serum PON1 activity analyses in standard biochemical tests, like other liver enzymes, may be effective in assessing the degree of liver failure and the degree of fatty liver, not only providing diagnosis. In the future, screening for PON1 status prior to illness may be very useful, particularly to identify individuals with major risk factors or to investigate the effects of OP exposure for agricultural workers.

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3 Erythrocyte Acetylcholinesterase as a Biomarker of Environmental Lead Exposure

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Abstract

Lead is a prime, ubiquitous, environmental toxicant and multi-organ poison. One of the most recognized deleterious effects of lead exposure is neurotoxicity, which may at least in part arise from perturbation in cholinergic neurotransmission with possible impact on acetylcholinesterase activity. Although primarily located at the neuromuscular junction and cholinergic brain synapses, this pivotal enzyme is also present in peripheral cells such as human erythrocytes. Erythrocyte acetylcholinesterase, which correlates positively with brain acetylcholinesterase and represents neurotoxic targets in brain, is a conventional biomarker for the neurotoxic effects of pesticide exposure. However, recent reports have unveiled the sensitivity of this extra-neural enzyme to other environmental contaminants particularly lead, bringing to attention its relevance in the assessment of environmental lead exposure and lead-induced neurotoxicity. This chapter summarizes the evidence of the remarkable diversity of erythrocyte acetylcholinesterase as a biomarker of environmental lead exposure and lead-induced alterations in human cholinergic system as well as the possible factors surrounding its applicability in this regard.

Keywords

Cholinergic neurotransmission · Environmental lead exposure · Erythrocyte acetylcholinesterase · Environmental contaminant · Lead · Lead-induced neurotoxicity · Multiorgan poison · Neurotoxic effects · Toxicant · Toxic metal

Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
BChE	Butyrylcholinesterase
BLL	Blood lead level
CNS	Central nervous system

LIN Lead-induced neurotoxicity
Pb Lead

Introduction

Lead (Pb) is a prime toxic environmental contaminant and multi-organ poison that has been known since antiquity (Anetor et al. 2016; Nwobi et al. 2021). Lead occurs naturally in ores with other metals but released into the environment during mining or processing of these ores (Hsu and Sabatini 2019). This heavy metal, although toxic, has some unique physico-chemical characteristics that guarantee its applicability in various products such as protection equipment, electrical equipment, paint, petrol, cosmetics, jewellery, and toys, among others (WHO 2021). The various anthropogenic activities, the uncontrolled involvement of lead in products as well as the unavailability of natural breakdown mechanisms for this toxic metal, have culminated to its continuous widespread environmental contamination as well as concomitant increased human exposure and attendant health risks. Although environmental lead exposure is a significant health concern of global magnitude, it is more common in fast industrializing countries with large chemical burden and developing countries that have weak or unimplemented environmental regulations and inadequate product content control and policies (Obeng-Gyasi 2019).

Environmental lead exposure from different sources occurs through routes such as ingestion, inhalation as well as dermatological contact with lead-contaminated products (Al Osman et al. 2019). However, following absorption of lead into the bloodstream, it is distributed to almost all major organs' systems where it induces different types of toxic effects of which the neurotoxic effect is considered to be of major concern and the most deleterious (Anetor et al. 2002; Anetor et al. 2008; Nwobi et al. 2019a). Yet, reliable and sensitive methods for predicting and assessing neurotoxicity remain a challenge to neuroscientists and toxicologists.

Lead-induced neurotoxicity (LIN) may manifest as perturbation of cholinergic neurotransmission that may present as alteration in the activity of acetylcholinesterase (AChE): a key enzyme that hydrolyses the neurotransmitter acetylcholine to ensure nerve impulse intermittency (Ortega et al. 2021). Although this enzyme is primarily located at the neuromuscular junction and cholinergic brain synapses, it can also be found at peripheral cells such as the human erythrocytes (Nwobi et al. 2019a; Felsztyna et al. 2020). Several reports have shown that erythrocyte AChE has several similar characteristics with neuronal AChE, correlates positively with brain AChE, and reflects neurochemical targets in the brain (Nehru and Sidhu 2001; Lionetto et al. 2013; Gupta et al. 2015; Nwobi et al. 2019a).

Although erythrocyte AChE has long been known as a biomarker for the neurotoxic effects of pesticide exposure (Assis et al. 2018), emerging reports over the past few years have shown that this enzyme is also sensitive to other environmental contaminants such as the toxic metals (Frasco et al. 2005; Phyu and Tangpong 2014; Fu et al. 2018). In line with this, the relevance of erythrocyte AChE in the assessment of human environmental lead exposure and LIN has continued to gain attention over

the years (Ademuyiwa et al. 2007; Khan et al. 2009; Gupta et al. 2015; Nwobi et al. 2019a). This chapter summarizes the available evidence of the remarkable diversity of the involvement of erythrocyte acetylcholinesterase as a biomarker of environmental lead exposure and lead-induced alterations in the human cholinergic system.

Chemistry, Forms, and Properties of Lead

Lead is a naturally occurring element with the chemical symbol Pb, derived from its Latin name, *plumbum*. Lead has an atomic number of 82 and a relatively high atomic weight of 207.2, making it a heavy metal. It has four stable isotopes: ^{208}Pb (51 to 53%), ^{207}Pb (20.5 to 23%), ^{206}Pb (23.5 to 27%), and ^{204}Pb (1.35 to 1.5%) (ATSDR 2020). Lead is a member of group 14 or p-block, subgroup IVA, and period 6 of the periodic table of elements. The 82 electrons in a lead atom have the shell structure of 2.8. 18.32. 18.4 and the ground state electron configuration of [Xe] 4f14 5d10 6 s2 6p2. As such, the valence shell of the lead atom in the ground state has two "s" and two "p" electrons. Notably, the two "s" electrons are resistant to ionization and are thus sometimes referred to as inert pair, making lead to be considered to have a stable oxidation state or valency of +2 (ATSDR 2020). Lead has a bluish-white color when freshly cut, develops a dull grayish color when exposed to air, and has a shiny chrome-silver luster, when it is melted into a liquid. It has a density of 11.34 g/cm^3, low melting point of 327.46° C, and boiling point of 1,750 °C to 1,755 °C (ATSDR 2020).

Lead constitutes 0.002% of the Earth's crust and exists naturally in ore with other metals and not in the elemental state (Hsu and Sabatini 2019). The main lead ore is galena (lead sulfide) (WHO 2010). Others include anglesite (lead sulfate), cerussite (lead carbonate), mimetite (lead chloroarsenate), and pyromorphite (lead chlorophosphate) (WHO 2010). Lead can also occur in other forms such as organic lead as found in tetra-ethyl lead used as additive in petrol (Galadima et al. 2012), or inorganic lead as occurs in old paint, soil, dust, and various consumer products.

Lead has a range of unique properties, which make it invaluable in various domestic and industrial applications. These include low melting point, high resistance to corrosion and fire, ability to absorb radiation, and sounds and other vibrations as well as being soft, malleable, and a relatively poor conductor of electricity (WHO 2021).

Sources and Routes of Environmental Lead Exposure

Human exposure to environmental lead was low prior to the industrial revolution. However, in recent years, it has risen because of various human activities such as industrialization, large-scale mining, uncontrolled applications of lead to products, and the continuous use of such lead products, all of which are coupled with a lack of environmental regulation and product content control and policies. This makes environmental lead exposure a problem of serious concern.

Several potential sources of lead exposure exist. However, the various sources and their relative importance may differ both within and between countries and

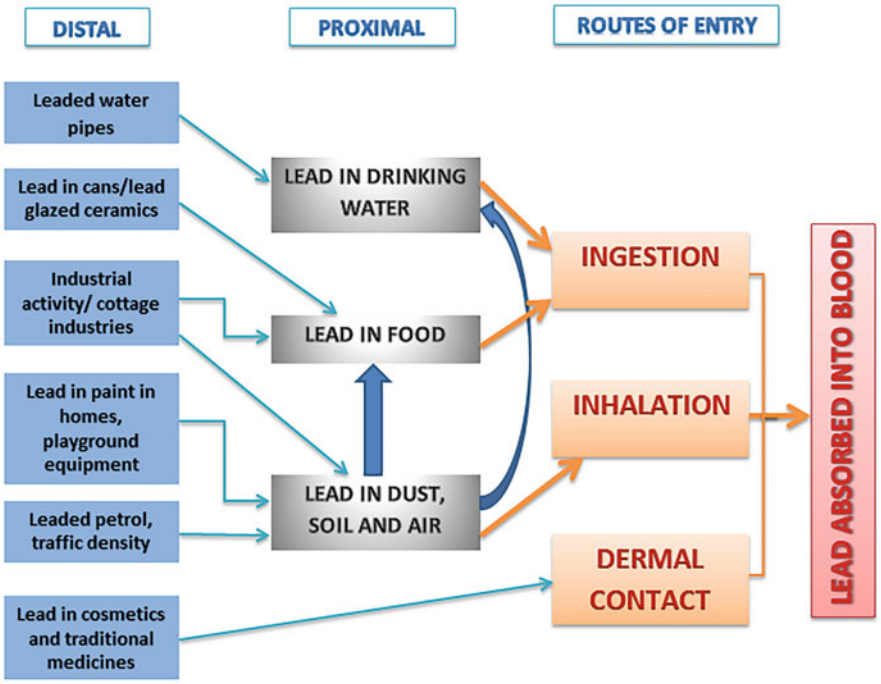

Fig. 1 Major sources and routes of environmental lead exposure. (Adapted from WHO 2021)

regions (Obeng-Gyasi 2019; WHO 2021). Nevertheless, some of the major sources of lead include leaded paint, lead emissions from industries, leaded water pipes and fittings, lead-glazed food vessels, lead-containing traditional folk medicine and cosmetics (WHO 2021) (Fig. 1).

Although the recognized routes of lead exposure include ingestion, inhalation, and dermatological contact, ingestion and inhalation have been reported to be the most significant routes (Fig. 1) (WHO 2021). Numerous cases of oral lead poisoning emanate from regularly ingesting small amounts of lead-contaminated substances such as dust or dirt, flakes of lead paint, food, water, and traditional remedies, among others. Young children, however, are more vulnerable to oral lead poisoning than adults are because they spend so much time in one place, play on the ground, have frequent hand-to-mouth contact, and eat objects that may contain or be contaminated with lead (WHO 2010).

Inhaling lead fumes or particles work is a common occupational route of exposure, but it can also happen at home if there is lead-contaminated airborne dust Exposure through inhalation of lead-contaminated air, dust or fumes is quite common in occupational settings but may also occur within the domestic environment (Pelclová et al. 2016). Dermal exposure can occur in the workplace or through the use of lead-containing cosmetics, but it is usually regarded as a minor and limited method of lead uptake (WHO 2021).

Toxicokinetics of Lead

Absorption, Distribution, and Elimination of Lead

The absorption of lead from the gastrointestinal tract is affected by several factors such as age, nutritional status, genetic factors, and the type of lead involved (WHO 2021). The absorption rate of ingested lead in adults is low (3–10%), compared with children, who have a higher rate (40–50%) (WHO 2021). Lead absorption can also be increased by fasting as well as nutritional deficiencies of important minerals such as calcium, zinc, and iron (Kordas et al. 2018; Rădulescu and Lundgren 2019).

Absorption of particulate lead by inhalation is dependent on particle size, concentration, and ventilation rate and is worthy to note that children may have higher exposure because of their increased air intake per unit of body weight compared to adults (WHO 2010). It has been reported that while small particles of lead (<1 μm) are deposited in the lower respiratory tract, from where they are almost entirely absorbed, larger particles (1–10 μm) are deposited in the upper airways, transferred by mucociliary transport to the esophagus and swallowed (WHO 2021). Dermal absorption is dependent on the skin's integrity and the lead's physicochemical qualities; it is minimal for inorganic lead and much greater for organic lead compounds (ATSDR 2020).

As soon as absorbed, lead gets to its first receptacle – blood, where about 99% is bound to erythrocytes, it is distributed to soft tissues such as the kidney, heart, and brain as well as the calcified tissues such as the bone – which serves as the major repository of lead (Fig. 2). Blood and soft tissues represent the active pool and bone the storage pool (ATSDR 2020). The blood lead level (BLL) represents recent exposure to lead from exogenous sources. It could also reflect lead redistributed from skeletal stores if there had been previous exposure to lead. This may be particularly important in pregnancy, when stored lead is released because of bone turnover (Osorio-Yáñez et al. 2021). About two-third of inorganic lead is excreted in the urine and the other one-third secreted in bile, into the intestine, and then excreted through the feces (Charkiewicz and Backstrand 2020). Lead is eliminated in two phases after exposure: The first (elimination from blood and soft tissues) takes about 20–30 days, and the second (slow elimination from the blood) involves excretion from the bones (Charkiewicz and Backstrand 2020). In general, the lead elimination half-life from blood and soft tissues is approximately 30 days while that of bone is 10–20 years; as a result, lead can be eliminated at an exceedingly slow rate, facilitating its accumulation in the body with more propensity to toxicity.

Toxicodynamics of Lead

Lead does not have any physiological function but induces a variety of toxicities ranging from subclinical to clinical toxicities with overt signs and symptoms. No safe threshold for lead exposure or blood lead level has been reported thus far, implying that the ideal blood lead level is zero although achieving this limit is rarely

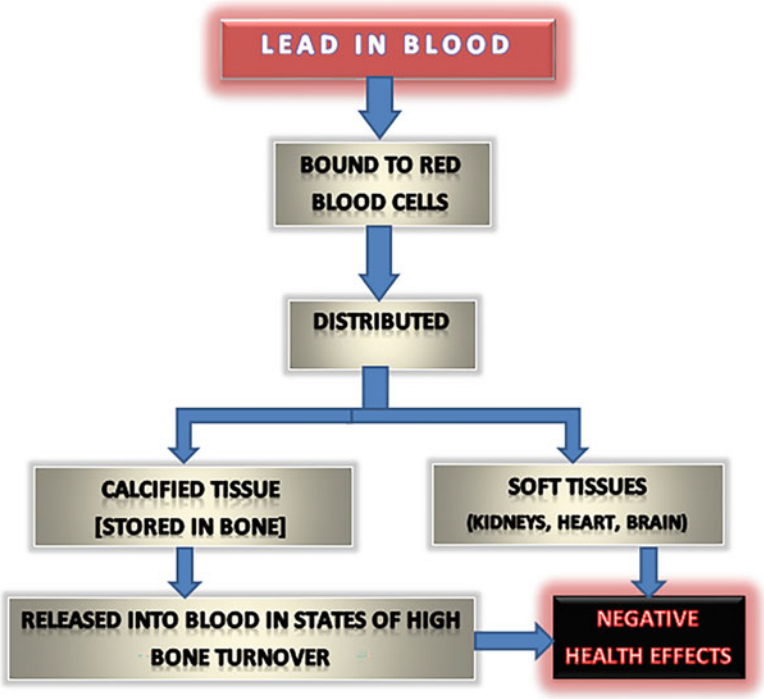

Fig. 2 Distribution of lead. (Adapted from WHO 2021)

feasible (Vorvolakos et al. 2016; ATSDR 2020; Ruckart et al. 2021). This may explain why no reference range for blood lead exists, but acceptable level (ACCLPP 2012). Lead has the ability to affect nearly all biochemical components, organs, and systems in the body. The principal systems affected may include among others the cardiovascular, hematological, renal, reproductive, skeletal, and neurological systems (Table 1).

Lead and Hematological Toxicity

One of the most well-studied systems on which lead has profound deleterious effect is the hemopoietic system, particularly its effects on the hem biosynthethic pathway – a pathway that has served as a surrogate for the diagnosis of lead poisoning (Patharkar et al. 2019). Three important enzymes in this pathway are downregulated by lead. The enzymes are aminolevulinic acid synthetase which catalyzes the formation of δ-aminolevulinic acid, δ-aminolevulinic acid dehydratase which catalyzes the formation of porphobilinogen from δ-aminolevulinic acid, and ferrochelatase which catalyzes the insertion of iron into protoporphyrin to form hem. Out of these 3 enzymes, δ-aminolevulinic acid dehydratase has been reported to be

Table 1 Lead toxicity and effects

Lead toxicity	Effects	Selected references
Lead and hematological toxicity	Inhibition of hem biosynthetic pathway key enzymes; aminolevulinic acid dehydratase, aminolevulinic acid synthetase, and ferrochelatase Decrease in life span of circulating erythrocytes through cell membrane fragility Anemia	Patharkar et al. (2019) Qader et al. (2021) WHO (2021)
Lead and renal toxicity	Hyperuricemia, gout, decreased renal clearance, tubular reabsorption, and glomerular filtration rate Increased risk of nephropathy and renal failure	Jung et al. (2019) Nakhaee et al. (2019)
Lead and cardiovascular disease	Hypertension Coronary heart disease, stroke, and peripheral arterial disease	Gambelunghe et al. (2016) Chowdhury et al. (2018), Obeng-Gyasi et al. (2018)
Lead and reproductive toxicity	Infertility in men and women Reduced sperm count Reduced fetal growth, decreased birth weight, preeclampsia, preterm birth and spontaneous abortion, and teratogenic effects	Ma et al. (2019) Wu et al. (2012) Dutta et al. (2021) WHO (2021)
Lead and bone metabolism	Alteration of essential bone minerals such as calcium and zinc Reduction of bone mineral content and the mechanical characteristics of long bones Alteration in biomarkers of bone turnover	Nwobi et al. (2019) Qi et al. (2020) Olchowik et al. (2014), Rodríguez and Mandalunis (2018) Nwobi et al. (2021)
Lead and neurotoxicity	Decreased cognition, intelligence quotient and behavior scores, alterations in attention, changes in visual-motor, reasoning skills, and impaired reading ability in children Increased rates of malaise, amnesia, headache, fatigue, lethargy, irritability, dizziness, and weakness in occupationally exposed adults Reduction in nerve conduction velocity, sensory and motor neuropathies	Crump et al. (2013) ATSDR (2020), Reuben et al. (2020) WHO (2021)

the most sensitive to lead (Qader et al. 2021). Lead also shortens the life span of circulating erythrocytes by making cell membranes more fragile (WHO 2021). The combined effects of lead on erythrocyte cell membranes as well as inhibition of enzymes involved in hem-biosynthesis may lead to anemia (WHO 2021).

Lead and Renal Toxicity

Lead induces proximal tubular injury in the kidneys, which manifests as proximal tubule nuclear inclusion bodies that may lead to tubulo-interstitial disease and fibrosis. Hyperuricemia and gout are also common findings associated with chronic lead toxicity (Jung et al. 2019). This may emanate from isolated proximal tubular

defects leading to increased tubular reabsorption and decreased secretion of uric acid. Proximal tubular defects may also lead to decreased renal clearance, tubular reabsorption, and glomerular filtration rate (Jung et al. 2019). These may predispose to increased risk of nephropathy and related renal failure (Nakhaee et al. 2019).

Lead and Cardiovascular Disease

In addition to hypertension (Gambelunghe et al. 2016), epidemiological reports have linked lead exposure with various cardiovascular-related clinical outcomes such as cardiovascular disease mortality, coronary heart disease, stroke, and peripheral arterial disease (Chowdhury et al. 2018; Obeng-Gyasi et al. 2018).

Lead and Reproductive Toxicity

Lead causes reproductive malfunction and infertility in men and women (Ma et al. 2019). Reduced sperm count in male partners of infertile couples has been associated to increased lead levels in seminal fluid (Wu et al. 2012). Maternal exposure, even to low lead levels, has been linked with reduced fetal growth, decreased birth weight, preeclampsia, preterm birth, and spontaneous abortion as well as teratogenic effects (Dutta et al. 2021).

Lead and Bone Metabolism

Lead exposure alters the metabolism of important essential minerals involved in skeletal metabolism such as calcium and zinc. Lead and calcium share similar metabolic characteristics because of their comparable biochemical nature as divalent cations (Godwin 2001). However, compared to calcium, lead has larger ionic radius, higher electronegativity and uneven charge distribution in the electron cloud, thereby, allowing it to bind to protein-binding sites with greater affinity than calcium and impairing physiological functions such as bone mineralization (Godwin 2001; Nwobi et al. 2019b). In the same vain, zinc, another divalent cation, which has stimulatory effect on osteoblastic bone formation and mineralization, could also be altered by lead (Qi et al. 2020). The perturbation in the metabolism of these essential bone minerals may manifest as reduction in bone mineral content and the mechanical characteristics of long bones (Olchowik et al. 2014; Rodríguez and Mandalunis 2018). Furthermore, it could cause a disruption in bone turnover by generating an imbalance in the dual processes of bone formation and resorption (Nwobi et al. 2021).

Lead and Neurotoxicity

When compared to other organ systems, the nervous system is the most susceptible and the major target for lead toxicity (Fang et al. 2021). Chronic lead exposure can

induce subtle alterations in neurological function in children and adults (Vlasak et al. 2019). Young children, on the other hand, are more vulnerable due to their high rate of lead absorption and higher penetration of lead through the blood-brain barrier, which is the most sensitive to damage (Nwobi et al. 2019a; WHO 2021). Children may develop neurological and cognitive sequelae such as decreased cognition, intelligence quotient and behavior scores, alterations in attention, changes in visual-motor and reasoning skills and impaired reading ability, and antisocial behavior, which may persist into adulthood (Crump et al. 2013; ATSDR 2020).

Increased rates of malaise, amnesia, headache, fatigue, lethargy, irritability, dizziness, and weakness have been reported in occupationally exposed adults, which may become more obvious and life-threatening in old age (ATSDR 2020; Reuben et al. 2020). These workers have also been reported to have reduced nerve conduction velocity, sensory and motor neuropathies, and wrist drop and/or foot drop (WHO 2021).

Cholinergic System

The cholinergic system plays a pivotal role in several CNS functions including cognition (Manzo et al. 1995). The dysfunction of this system has been reported to be responsible for the behavioral disturbances and learning and memory deficits in humans and animals (Nehru and Sidhu 2001). Several studies have suggested that the alteration in cholinergic neurotransmission involving the cholinesterases may be responsible for the neurotoxic effects of lead (Bressler and Goldstein 1991; Goldstein 1992; Anetor et al. 2002). This implies that the disrupted biochemical intracellular communication may occur before the well-known, overt clinical signs of LIN.

Cholinesterases are enzymes that hydrolyze serine and have a high affinity for choline esters. Varieties of cholinesterase enzymes with different properties exist in the animal tissues; however, the two main types are acetylcholinesterase (AChE, EC 3.1.1.7), also known as true cholinesterase, and butyrylcholinesterase (BChE, EC 3.1.1.8), also known as pseudocholinesterase (Hajjawi 2012; Rosenberry et al. 2017). Although the three-dimensional structures of AChE and BChE are strikingly similar, they differ in the amino acids that line a deep, narrow gorge at the bottom of which a catalytic site is located. In human BChE, there is replacement of 6 of the 14 aromatic amino acids that line AChE's gorge with aliphatic amino acids. As a result, BChE has a larger acyl pocket than AChE but lacks the peripheral site found in AChE (Hajjawi 2012; Rosenberry et al. 2017).

The main and most known function of AChE is to hydrolyze acetylcholine, a major neurotransmitter and neuromodulator, into choline and acetic acid, thereby, modifying nerve impulses involved in neural communication (Huerta-Ocampo et al. 2021). Butyrylcholinesterase, on the other hand, hydrolyzes butyrylcholine more efficiently than acetylcholine and is commonly engaged in exogenous chemical detoxification and bioactivation (Hajjawi 2012).

Acetylcholinesterase

In its basic working state, AChE is a dimmer amphipathic protein with a molecular weight of 160 K and a single inter-subunit disulfide bond (Rosenberry and Soggin 1984). Acetylcholinesterase is a fast-acting, uncompetitive enzyme with a turnover rate of 10^3 to 10^4 s^{-1} and a catalytic rate around the diffusion limit (Quinn 1987). Acetylcholinesterase hydrolyzes acetylcholine, a key neurotransmitter, thereby, ensuring nerve impulse intermittency. Apart from being primarily found in neuromuscular junctions and cholinergic brain synapses, AChE is also found in peripheral tissues such as the human erythrocytes, where it is firmly attached (Felsztyna et al. 2020).

X-ray crystallographic research of acetylcholinesterase revealed that catalysis occurs in a 20-Å deep active site gorge comprising a catalytic triad of serine, histidine, and glutamate residues located at the base of the active site, denoted as the cholinergic site, acylation, or A-site. Acetylcholinesterase has another distinct region near the rim of the gorge or enzyme surface, called the peripheral site or P-site (Soreq and Seidman 2001; Johnson et al. 2005; Rosenberry et al. 2017) (Fig. 3).

The P-site is lined with aromatic residues that are pivotal in the binding and orientation of aromatic and/or cationic substrates as they move from the P-site to the A-site (Rosenberry et al. 2017). The P-site has been reported to serve as an intermediate binding site for cationic ligands such as lead, which, thereafter, proceed to the acylation site (Johnson et al. 2005; Rosenberry et al. 2017). Binding to P-site can result in allosteric activity change of the acylation step, which inadvertently affects the whole rate of enzyme reaction. The activity of AChE reduces at high substrate levels, which by corollary may imply high lead level (Mallender et al. 2000; Rosenberry et al. 2017). This phenomenon known as substrate inhibition has been suggested to occur through steric blockade of product release caused by the binding of an additional substrate molecule to the P-site (Szegletes et al. 1998; Rosenberry et al. 2017). This implies that the binding of lead to the P-site of the enzyme molecule may result in the creation of a complex whose stability may give rise to varying degrees of alteration of enzyme-effective function with consequent possible conformational change of the enzyme (Auf der Heide 2007) (Fig. 3).

Extraction and Purification of Erythrocyte Acetylcholinesterase

A mature human erythrocyte also known as red blood cell is a non-nucleated, biconcave, disc-shaped cell filled with hemoglobin that transports oxygen and carbon dioxide between the lungs and tissues (Ciaccio et al. 2021). Acetylcholinesterase is a glycosylphosphatidylinositol-anchored protein that is firmly connected to the outer membrane of the human erythrocyte by hydrophobic and hydrostatic forces (Felsztyna et al. 2020). The catalytic efficiency of erythrocyte AChE is determined by its amphipathic extraction and purification medium such as detergents like Triton X-100 and sodium chloride (0.14 M) (Gupta et al. 2015). The role of sodium chloride is to break the electrostatic interactions with membrane proteins that protect some of

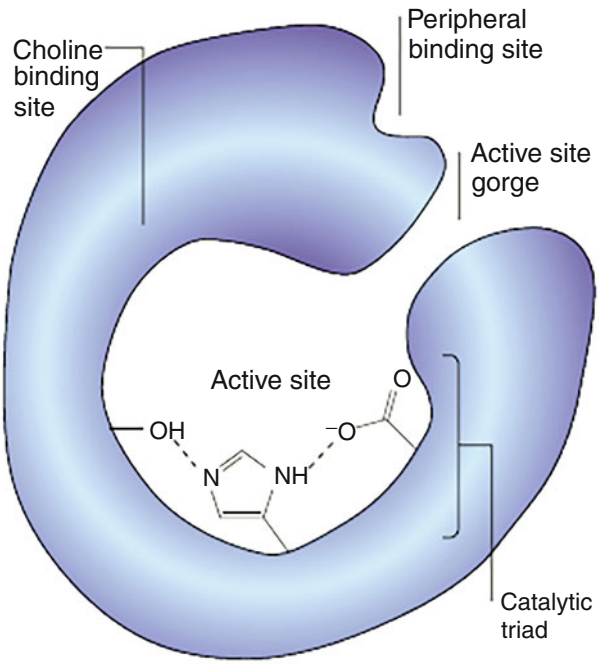

Fig. 3 Structural features of the acetylcholinesterase enzyme. The cholinergic site is at the bottom of a deep, narrow gorge while the peripheral binding site is near the rim of the gorge or enzyme surface. The amino acids serine 203, histidine 447, and glutamate 334 form a catalytic triad in human acetylcholinesterase. (Reproduced with permission from Soreq and Seidman (2001))

the enzymes from detergent action (Gupta et al. 2015). This allows the detergent to attack the membrane more efficiently and solubilize more membrane-bound enzymes.

The biochemical assay of acetylcholine involves the hydrolysis of acetylthiocholine to form sulfhydryl groups which react with Ellman reagent (5,5′-dithiobis (2-nitrobenzoic acid)) to generate a yellow color detected at the wavelength of 405 nm, whose intensity is proportional to choline and by implication, AChE activity (Ellman et al. 1961). A clinical assay involves the hydrolysis of acetylcholine by AChE to form choline and acetic acid; the change in pH due to the liberation of acetic acid is measured using metanitrophenol - an indicator, whose intensity of yellow color in alkaline solution decreases as acetic acid concentration is increased (Hajjawi 2012).

Erythrocyte Acetylcholinesterase in Biological Monitoring of Environmental Lead Exposure

Environmental lead exposure in association with its health risks, such as LIN, is an issue of serious concern. Hence, there is need for a continuous, accessible, reliable and sensitive monitoring of lead exposure particularly in the developing

countries where exposure to lead recently appears more intense (Attina and Trasande 2013; Obeng-Gyasi 2019; WHO 2021). The lack of both human CNS cells or tissues and sensitive neurochemical indicators as well as ethical and practical considerations are the principal reasons the majority of the human research on LIN in the past relied greatly on neurobehavioral tests or electrophysiological measurements (Seppalainen 1988; Anger 1990; Manzo et al. 1996). Although these methods may be highly valuable in the clinical setting as useful metrics in detecting/diagnosing behavioral changes and neurological disorders, they are unable to identify specific nervous system components affected by the neurotoxicant (Ademuyiwa et al. 2007). However, these limitations have been overcome through the use of direct biochemical methods involving promising peripheral alternatives that are not only present in more easily and ethically obtainable body fluids such as blood, but also exhibit biochemical signals of neurotransmission similar to those involved as neurotoxic targets in the CNS (Manzo et al. 1995; Manzo et al. 1996; Ademuyiwa et al. 2007). Thus, the extraneuronal enzyme – erythrocyte AChE, which is a prime candidate in this case – may be considered to be very useful in assessing exposure and response of the CNS when functional damage may not yet be apparent – silent neurotoxicity (Nwobi et al. 2019a). Several reports have shown that erythrocyte AChE exhibits similar characteristics with those of neuronal enzyme, correlates positively with brain AChE, and reflects neurochemical targets in the brain (Nehru and Sidhu 2001; Ademuyiwa et al. 2007; Lionetto et al. 2013; Gupta et al. 2015).

Blood Lead Level Versus Erythrocyte Acetylcholinesterase in Lead-Induced Neurotoxicity

Although many sample matrices such as blood, bone, tooth, hair, nail, saliva, and urine have been studied, the most used biomarker of lead exposure is the blood lead level (BLL) (Sommar et al. 2014). However, BLL has been considered insufficiently sensitive owing to some limitations such as poor response to changes at increased lead exposure, as well as exhibition of large inter-individual difference in health response to a particular lead level. Blood lead level also has a short half-life of approximately 30 days; hence, it primarily reflects current exposures and inadequately assesses long-term risk such as LIN, which has a more insidious impact (Nwobi et al. 2019a). As a result, the early detection of LIN appears to be a significant concern of global magnitude and a critical area of research, thus necessitating a continuing search for its accessible and inexpensive biomarkers. Interestingly, erythrocyte AChE is promising in surmounting these limitations because it has a longer half-life of 2–3 months and its activity appears to be altered in LIN (Majidi et al. 2018).

Reports from animal models, humans, and in vitro investigations exist on the use of AChE as a surrogate biomarker for lead exposure (Ademuyiwa et al. 2007; Ani et al. 2007; Reddy et al. 2007; Khan et al. 2009; Gupta et al. 2015; Nwobi et al. 2019a). Lead has been reported to directly affect AChE activity in different brain regions such as the hippocampus, cerebellum, cortex, and midbrain, resulting in

alteration in motor coordination activity and perturbation in cognitive behavior (Reddy et al. 2003; Ani et al. 2007; Reddy et al. 2007). A remarkable observation by these investigators is that the perturbation of AChE activity by lead persists even after the cessation of the stimulus exposure, underscoring the strength of the biological association between AChE and lead.

In an in vitro study by Gupta et al. 2015, the effect of various doses of lead on human erythrocyte AChE activity showed strong inhibition of the enzyme in a time-dependent, uncompetitive manner (Gupta et al. 2015). This report also underscores the neurological far-reaching implication of lead exposure on AChE activity and biomarker of effect potential.

Ademuyiwa et al. 2007, reported lead inhibition on the activity of erythrocyte AChE in adult artisans occupationally exposed to lead in Abeokuta, Nigeria. The investigators suggested that erythrocyte AChE could be employed as a putative biomarker for lead exposure, specifically lead-induced cholinergic system alterations in individuals living in a lead-contaminated environment (Ademuyiwa et al. 2007). In the same vein, Khan et al. 2009, investigated erythrocyte and plasma AChE activity in adult painters in Lucknow city, India, and reported that lead exposure, assessed by increased BLL, disrupted cholinergic function by remarkably inhibiting the erythrocyte AChE activity in the workers (Khan et al. 2009).

Nwobi et al. 2019a, investigated the potential role of BLL, erythrocyte AChE activity, and intelligence quotient in the early identification of LIN in apparently healthy children that had elevated BLL, an indication of environmental lead exposure based on ACCLPP 2012. However, their findings showed increased erythrocyte AChE activity which associated positively with BLL (Fig. 4) and negatively with intelligence quotient (Nwobi et al. 2019a). Thus, suggesting that erythrocyte AChE may be useful for the early identification of childhood lead exposure and LIN.

The Possible Mechanisms Surrounding the Interference of Lead with Erythrocyte Acetylcholine Activity

The mechanisms surrounding the interference of lead with erythrocyte AChE activity so far appear to be incompletely elucidated, and no one unifying molecular explanation has been proposed to explain it. As a result, various research groups have proposed or speculated on plausible molecular pathways. Some investigators have associated the altered erythrocyte AChE activity in lead exposure to the effect of free radicals generated by lead (Tsakiris et al. 2000; Khan et al. 2009). Although some authors have linked the altered AChE activity to the effect of binding of lead to the thiol group in the enzyme (Phyu and Tangpong 2014), others have argued that erythrocyte AChE does not contain free thiol groups in its structure to which lead could bind (Rosenberry 1975). However, most recently, the alteration in erythrocyte AChE activity has been linked to the displacement of nutritionally essential metals such as calcium, from their protein-binding sites by lead (Anetor et al. 2002; Sharma et al. 2015; Nwobi et al. 2019).

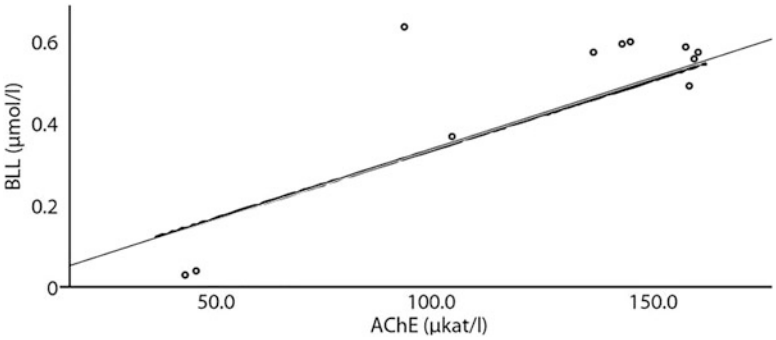

Fig. 4 Scatter plot showing linear relationship between blood lead level (BLL) and erythrocyte acetylcholinesterase (AChE) activity in children in Ibadan, Nigeria. (Reproduced from Nwobi et al. (2019a))

The structure of AChE may shed more light on the AChE-lead interaction. It has been reported that the tertiary structure of the EF-hand regions of AChE corresponds closely to the well-characterized EF-hand motifs of calcium-binding proteins such as calmodulin, suggesting that divalent cations may be critical to the folding, maintenance of structure, function, and activity of AChE (Tsigelny et al. 2000). Other divalent metals such as lead can also bind to EF-hand motifs of calcium-binding proteins (McPhalen et al. 1991). Lead, a toxic metal, at low concentration has been reported to increase the activity of calcium-binding protein, which on the other hand is inhibited at higher concentration of lead (Kasten-Jolly and Lawrence 2018).

This could explain at least in part, the lead-induced increase in erythrocyte AChE activity in school children reported by Nwobi et al. (2019), which appeared not to be in line with the findings of other researchers who worked on occupationally exposed adults but reported lead-induced inhibition of AChE activity (Ademuyiwa et al. 2007; Khan et al. 2009). This explanation seems plausible for two reasons. First, Nwobi et al. (2019) recruited children that were not occupationally exposed to lead compared to other researchers that employed occupationally exposed adult workers. Second, the mean BLL in the children recruited by Nwobi et al. (2019) was quite small, 0.4 µmol/L (8.3 µg/dL), compared to the mean BLL of occupationally exposed workers, 35 µg/dL and 21 µg/dL, reported by Ademuyiwa et al. (2007) and Khan et al. (2009), respectively. Reports have shown that the activity of AChE reduces at high substrate levels, which by corollary may imply high lead level (Mallender et al. 2000; Rosenberry et al. 2017). This may further explain why the erythrocyte AChE, which was exposed to low lead level compared to the occupationally exposed adults, positively correlated with the BLL in Nwobi et al. 2019a. However, these reports underscore the need for more population-based studies to compare the effect of low and high level of lead exposure on erythrocyte AChE in children and adults.

Application of Erythrocyte Acetylcholinesterase to Prognosis

The application of erythrocyte AChE as a biomarker of environmental lead exposure may imply higher correlation with brain AChE activity and longer time of interaction with lead, permitting reliable detection of lead for a longer period after exposure. Hence, erythrocyte AChE could be considered an important promising index to determine the degree of environmental lead exposure and LIN as well as a monitoring alternative to BLL, a conventional biomarker of lead exposure. However, so far, it is not yet completely clear to what extent the data from erythrocyte AChE activity may be superior to the information obtained from the measurements of BLL regarding their usefulness as putative biomarkers of environmental lead exposure due to the following reasons:

1. There is inconsistent result on the erythrocyte AChE activity on exposure to low and high lead exposure, necessitating further research to identify low concentration cut-off at which lead activates the enzyme activity and high concentration cut-off at which the lead inhibits the enzyme activity.
2. Erythrocyte AChE also shows sensitivity to other environmental contaminants other than lead (Frasco et al. 2005; Phyu and Tangpong 2014; Fu et al. 2018), implying that the combinations of different environmental contaminants may exert additive or synergistic inhibitory effect on erythrocyte AChE activity. As a result, the changes in the activity of this enzyme may be interpreted as an integrative measurement of the overall neurotoxic risk posed by the load of all the bioavailable environmental contaminants rather than just lead.
3. Erythrocyte AChE activity may also be altered by some health conditions such as pregnancy, anemia, bleeding, and reticulocytosis, which may confound the evaluation and interpretation of enzyme activity (Auf der Heide 2007).

Clearly, it is pertinent to consider or resolve the foregoing confounding issues before erythrocyte AChE can be accepted as an alternative biomarker that is superior to the use of BLL for the assessment of environmental lead exposure.

Application of Erythrocyte Acetylcholinesterase to Other Conditions

Erythrocyte AChE plays diverse significant roles in health and diseases (Saldanha 2017). This enzyme aids in the preservation of erythrocyte shape and size as well as serves as a marker of membrane integrity (Gupta et al. 2015). It is also involved as the Yt antigen of the Cartwright blood group and has been linked to the expression of hemoglobin (Bartels et al. 1993).

The activity of erythrocyte AChE declines as erythrocytes age, and it likewise reduces with age in people as oxidative stress rises in response to increasing age (Prall et al. 1998; Saldanha 2017). Erythrocyte AChE enzyme activity increases in healthy females compared to males (Hilário et al. 2003; Saldanha 2017). These

gender-related changes in the enzyme's activity and the fluidity of the membrane hydrophobic area under the impact of adrenaline may explain, at least in part, the differences in responses, attitudes, and behaviors under stress circumstances between men and women (Hilário et al. 2003; Saldanha 2017). These differences may hold some useful promise in the management of diseases and medication reactions, at least at the cellular level.

Increased erythrocyte AChE activity has been linked with Parkinson's disease, essential hypertension, glaucoma, retinal vasculitis, amyotrophic lateral sclerosis, and Hirschsprung's disease, implying that this enzyme is significantly involved in inflammation (Silva-Herdade and Saldanha 2013; Saldanha 2017). Decreased erythrocyte AChE activity has been reported in patients with type 1 diabetes as well as individuals with paroxysmal nocturnal hemoglobinuria, which put them at risk of complement system lysis (Suhail and Rizvi 1989; Ueda et al. 1990). Decreased erythrocyte AChE activity has also been observed in farmers that are exposed to pesticides. This could result from the direct harmful effects of the pesticides on the integrity of the erythrocyte membrane (Lozano-Paniagua et al. 2016). It is also noteworthy that there is the need to rule out these confounders while considering erythrocyte AChE as a promising and reliable biomarker of environmental lead exposure or LIN.

Mini-Dictionary of Terms

- Acetylcholine: A major neurotransmitter in the cholinergic system.
- Acetylcholinesterase: A cholinergic enzyme (also known as true cholinesterase) that rapidly hydrolyzes acetylcholine into acetic acid and choline, thereby terminating its action and ensuring the intermittence of nerve impulses.
- Blood lead level: The most commonly used biomarker of lead exposure that represents absorbed doses of lead.
- Central nervous system: The part of the nervous system that consists of the brain and spinal cord as well as coordinates the activity of the entire nervous system.
- Erythrocyte: A red blood cell, which in humans, is a biconcave, nonnucleated disc that contains the pigment hemoglobin, which imparts the red color to blood as well as transports oxygen and carbon dioxide to and from the tissues.
- Lead: A heavy metal that is a prime ubiquitous environmental and occupational toxicant as well as a multiorgan poison.
- Lead-induced neurotoxicity: The alteration of the normal activity of the central nervous system or damage to it, because of exposure to lead.

Key Facts of Environmental Lead Exposure

- Lead is a prime ubiquitous environmental contaminant as well as a multiorgan poison.
- Major routes of lead exposure include ingestion, inhalation, and dermatological contact.

- Blood lead level is the most commonly used biomarker of lead exposure.
- The contemporary population's body lead burden is 500–1000 times more than that of their preindustrial forebears (WHO 2010).
- Lead exposure is responsible for 900,000 deaths, loss of 21.7 million years of healthy life, 62.5% of developmental intellectual disability of unclear etiology, 8.2% of hypertensive heart disease, 7.2% of the ischemic heart disease, and 5.65% of stroke in the world (IHME 2019).
- The global cost of lead exposure in children is projected to be $977 billion per year or 1.20% of global GDP (Attina and Trasande 2013).

Key Facts of Erythrocyte Acetylcholinesterase as a Biomarker of Environmental Lead Exposure

- Lead-induced neurotoxicity is the most deleterious effect of lead exposure and could manifest as alteration in cholinergic neurotransmission.
- Erythrocyte acetylcholinesterase is an extra neuronal enzyme that is similar to the brain acetylcholinesterase and represents neurochemical targets in the brain.
- The structure of this enzyme shows an acylation site for binding of acetylcholine and a peripheral site where lead could bind.
- The applicability of erythrocyte acetylcholinesterase activity as a biomarker of environmental lead exposure should be independently confirmed by the use of blood lead level or other molecular approaches.

Summary Points

- Lead is a prime, toxic, ubiquitous environmental contaminant and multiorgan poison that poses significant health challenge, particularly in developing countries, which also bear the brunt of the associated health and socioeconomic implications.
- One of the most recognized deleterious effects of lead exposure is neurotoxicity particularly in the central nervous system.
- Blood lead level is the commonly used marker of lead exposure but has been considered insufficiently sensitive owing to some limitations such as poor response to changes at increased lead exposure, exhibition of large inter-individual difference in health response to a particular lead level, and possession of a short half-life of approximately 30 days, which make it primarily reflect current exposures and inadequately assesses long-term risk such as led-induced neurotoxicity, which has a more insidious impact.
- However, the extraneural enzyme – erythrocyte acetylcholinesterase – appears to be an important tool to determine the degree of environmental lead exposure and lead-induced neurotoxicity because it implies higher correlation with brain acetylcholinesterase activity, represents neurochemical targets in brain, and has

longer time of interaction with lead, which allows reliable detection of lead for a longer period after exposure, unlike blood lead level.

- It is note-worthy that the applicability of erythrocyte acetylcholinesterase in this regard appears to have some confounding factors, which may interfere with the evaluation and interpretation of enzyme activity; it shows sensitivity to other environmental contaminants apart from lead, its activity may differ on exposure to low and high lead exposure, and it may be altered by some health conditions.
- It is pertinent to consider and resolve the above limitations before accepting erythrocyte acetylcholinesterase as a sufficiently reliable, specific, and sensitive biomarker for environmental lead exposure and lead-induced neurotoxicity.
- Hence, at the moment, although erythrocyte acetylcholinesterase appears to hold some promise as a useful biomarker of environmental lead exposure and lead-induced neurotoxicity, its activity should be verified independently using blood lead levels or other molecular approaches.

Cross-References

► Biomarkers of Lead Exposure: Platforms and Analysis

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Fortilin as a Biomarker in Toxicity

4

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Abstract

Fortilin, the multifunctional protein, also known as translationally controlled tumor protein (TCTP), presents in most eukaryotic organisms. It is involved in crucial cellular processes, such as cellular growth and apoptosis. Therefore, dysregulation of fortilin levels will negatively impact cells resulting in diseases and toxicities. Interestingly, fortilin has been investigated thoroughly as an antiapoptotic biomarker, but few studies have exposed this protein as a toxicity biomarker. This chapter attempts to collate the biological roles of fortilin for better understanding of its potential utility as a toxicity biomarker that could diagnose and provide prognosis in relevant cases. Along this line, different mechanisms, biological interactions, and foreseen applications of fortilin are described herein. In addition, future recommendations for scientific studies, particularly toxicological ones, are presented at the end.

Keywords

Fortilin · Biomarkers · Toxicity · TCTP protein · HRF protein · TPT1 protein · Tumor protein · Translationally controlled tumor protein · Histamine-releasing factor · Calcium-binding protein · Bromide · Methomyl · Environmental exposure · Human · Rat

Abbreviations

5-FU	5-Fluorouracil
AKT	Protein kinase B
BMPs	Multifunctional growth factors structurally belong to the TGFβ superfamily
Br^-	Bromide
Ca^{2+}	Calcium ion
cDNA	Complimentary DNA
CRISPR	Clustered regularly interspaced short palindromic repeats
DHA	Dihydroartemisinin
EAC	Ehrlich ascites carcinoma
EF1	Elongation factor 1
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
EsTCTP	Fortilin gene in the Chinese mitten crab (*Eriocheir sinensis*)
HCC	Hepatocellular carcinoma
HEMA	2-Hydroxy-ethyl methacrylate
HRF	Histamine-releasing factor
JNKs	c-Jun NH2-terminal kinases
K_d	Dissociation constant
MCL1	Myeloid cell leukemia sequence 1
MDM2	Mouse double minute 2 homolog
MIC-1	Macrophage inhibitory cytokine 1
Mst1	Serine/threonine kinase

Na, K-ATPase	Sodium-potassium ATPase
PAHs	Polycyclic aromatic hydrocarbons
Pm-fortilin	Fortilin from *Penaeus monodon*
PRX1	Peroxiredoxin-1
rFm-Fortilin (FL)	Fortilin from *Fenneropenaeus merguiensis*
rFortilin	Recombinant fortilin
RNAi	RNA interference
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
SD-TLHA	SD selective medium lacking tryptophan, leucine, histidine, and adenine
$siRNA_{fortilin}$	Small interfering RNA against fortilin
Src	Non-receptor protein tyrosine kinase
TCTP	Translationally controlled tumor protein
Tp-fortilin	Fortilin of *Trichinella pseudospiralis*
TPT-1	Tumor protein, translationally controlled 1
TSC-22	Transforming growth factor-β-stimulated clone-22
UPR	Unfolded protein response
WSSV	White spot syndrome virus
Y2H	Yeast two-hybrid system

Introduction

Fortilin or P23 is a multifunctional protein known as translationally controlled tumor protein (TCTP). Also called histamine releasing factor (HRF), it presents in most eukaryotic organisms (Bommer and Telerman 2020; Fujita et al. 2008; Susini et al. 2008). Figure 1 illustrates the different nomenclature of fortilin. It was first described as a growth factor-induced protein in murine cell lines (Bommer and Telerman 2020). The amino acid sequence of fortilin is highly conserved among species ranging from human to rice, most abundantly in the liver and kidneys (Graidist et al. 2007). Fortilin is involved in a range of cellular processes and homeostasis, such as promotion of growth and development (Bommer and Telerman 2020). In addition, it is involved in cellular response against general stress conditions such as heat stress (Bommer and Telerman 2020). Its cellular levels are highly regulated by variety of biological signals and molecules (Bommer and Telerman 2020). Therefore, dysregulation of fortilin is associated with apoptosis, different diseases, and toxicities. Here, we present significant insight on this important protein in an attempt to expose part of its potential use as a biomarker of toxicity.

Fortilin Characterization

Fortilin is a hydrophilic protein of 172 amino acids that presents intracellular in the nucleus as well as in the cytosol (Graidist et al. 2007). The amino acid polypeptide was found with 18–23 kDa and yet with no sequence similarity to any other

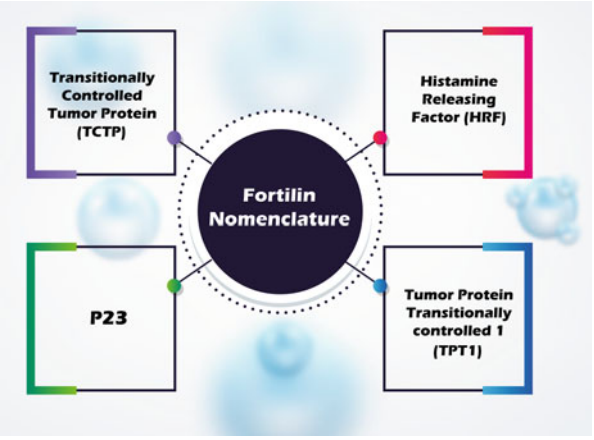

Fig. 1 Fortilin nomenclatures. This figure shows the different names of fortilin

sequenced proteins (Susini et al. 2008). Fortilin gene symbol TPT-1 was first described in yeast lines TMA19 or Mmi1 (Susini et al. 2008). Originally, fortilin was cloned as a human homologue to the mouse protein p21 (Li et al. 2001). Fortilin crystal structure had an interesting channel-forming helices similar to that of the proapoptotic protein Bax (Susini et al. 2008). Refer to Fig. 2 for further illustration of fortilin structure. The protein was discovered in Ehrlich ascites carcinoma (Ehrlich cells, EAC), a murine mammary adenocarcinoma model (Susini et al. 2008). Fortilin exists in various normal tissues with notably higher levels in cancer cell lines (Li et al. 2001). Further highlights on the functions and interactions of fortilin are upcoming to reveal its importance in normal and pathological conditions including toxicities.

Fortilin Functions

Fortilin is involved in a multitude of cell biological processes including stress response, signal transduction, apoptosis, and functioning of metabolic enzymes and different cellular components such as cytoskeleton, proteasomes, and nucleosomes (Bommer and Telerman 2020). A variety of biological functions of fortilin as well as its roles in response to stress are explained here and are summarized in Fig. 3.

Fortilin Roles in Cell Growth and Organ Development Process

The term fortilin is derived from the observations that fortilin transcripts accumulate in resting cells and upon cell requirements instantly translated into the protein (Susini et al. 2008). In another word, it is highly expressed in proliferating cells and very depleted in resting ones within variety of cellular processes in different organisms. For example, fortilin is involved in insect development through

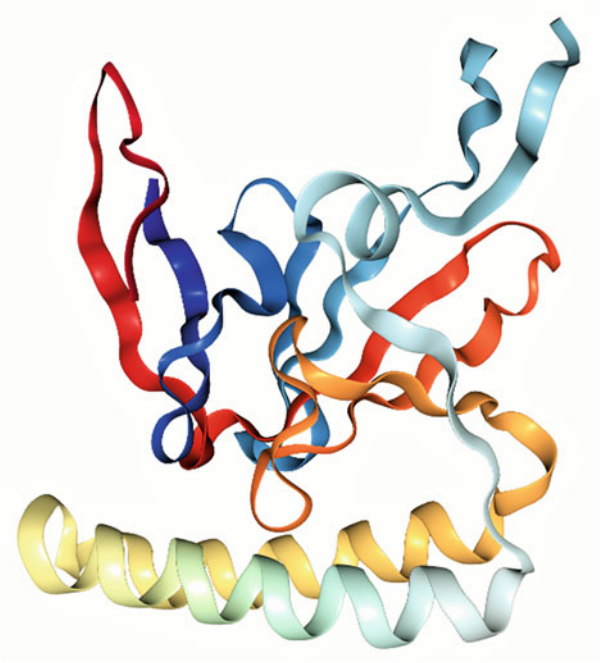

Fig. 2 Fortilin molecular structure. This figure shows a drawing of the human fortilin molecule (PDB id: 509 L). From the Protein Data Bank (Berman et al. 2000)

proliferation of epithelial cells (Kwon et al. 2019) and regulation of Rheb-GTPase activity (Susini et al. 2008); branching of plant roots through formation of lateral roots (Branco and Masle 2019); cell cycle progression through signalosome interaction with CSN4, a subunit of the COP9 signalosome complex that regulates Cullin-RING ubiquitin ligases to control the G1/S transition of the cell cycle (Bommer and Telerman 2020); tissue regeneration of stem cells and liver tissue (Lin et al. 2020) by activating mTORC2/AKT signaling for protein synthesis (Lin et al. 2020); brain development in mice by the process of axon guidance (Chen et al. 2020); and prevention of oocyte quality deterioration by regulating spindle assembly in the postovulatory aging of mouse oocytes (Jeon et al. 2017). On the other hand, fortilin dysregulation negatively affects growth. For instance, disruption of fortilin using CRISPR/Cas9 in the silkworm *Bombyx mori* resulted in developmental arrest and proliferation defects in the intestinal epithelium with subsequent larvae lethality (Kwon et al. 2019).

Regulation of Protein Synthesis and Degradation

Fortilin is involved in protein synthesis as well as degradation. For example, fortilin interacts with the elongation factors EF1 and other translational apparatus of proteins in human cells (Langdon et al. 2004). Fortilin activates protein

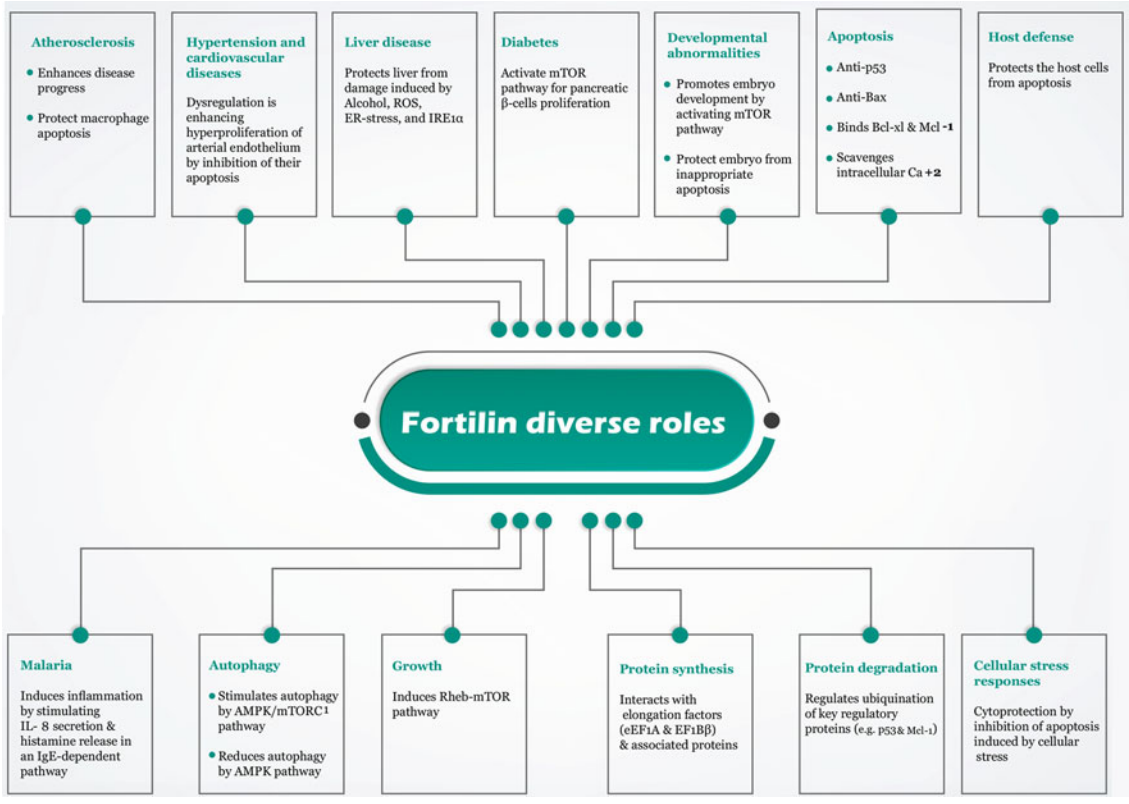

Fig. 3 Fortilin diverse roles. This is an infographic illustration of the variety fortilin biological roles in response to different stress, diseases, and its function in host defense, protein synthesis, and degradation

synthesis through mTORC2/AKT signaling to promote liver regeneration (Lin et al. 2020). In yeast, fortilin is involved in ribosomes functions and translational processes (Fleischer et al. 2006). On the contrary, fortilin promotes degradation of proteins such as the apoptotic protein p53. It inhibits mouse double minute 2 homolog (MDM2) auto-ubiquitination by binding to the p53-MDM2 complexes, resulting in MDM2-mediated ubiquitination and degradation of the protein p53 (Amson et al. 2012). Further details on this process will be presented later in the chapter.

Biological Stress Reactions and Autophagy

Fortilin is a reported antiapoptotic protein that protects cells in response to general stress conditions such as DNA damage in mammalian cells (J Zhang et al. 2017), plants (Betsch et al. 2017), and insects (Choi et al. 2017). Fortilin plays a role in the unfolded protein response (UPR), which is a process of endoplasmic reticulum (ER)-stress defense (Pinkaew et al. 2017). Once the protein overload in the ER becomes overwhelming, IRE1α (an ER-located transmembrane RNase) initiates the UPR by its protein kinase and endonuclease activities, eventually leading to apoptosis (Pinkaew et al. 2017). In addition, when fortilin binds to phosphorylated IRE1α, it prevents the activation of another apoptotic pathway named the c-Jun NH2-terminal kinases (JNKs) apoptosis pathway (Pinkaew et al. 2017). Furthermore, fortilin prevents ray-induced DNA damage; cells lacking fortilin failed to repair chromosomal damage induced by γ-rays through physical interactions between fortilin and ATM, an early sensor of DNA damage (J Zhang et al. 2017).

Fortilin's role in stress reactions was also demonstrated in nonmammalian systems. For example, fortilin promoted growth rate and photosynthesis of tobacco plants following exposure to a salty and osmotic stress environment (de Carvalho et al. 2017). Fortilin was reported as a cytoplasmic protein in the trypanosome's bloodstream that is involved in cell cycle regulation during heat-stress response (Jojic et al. 2018). Additionally, fortilin expression increased during the process of heat-stress adaptation of *Trichinella pseudospiralis* infective-stage larvae (Mak et al. 2007). Using gene knockout, studies suggested the implication of fortilin in cell survival mechanisms because fortilin-deficient mice (Chen et al. 2007) and fortilin-deficient *Drosophila* mutants (Choi and Hsu 2007) died early during embryogenesis. Figure 4 demonstrates the action of fortilin in response against different stress conditions.

Fortilin-ion Relationship

There is a number of reports on the presence of direct and indirect fortilin relationships with biological ions like sodium, potassium, and calcium. Further explanation is below.

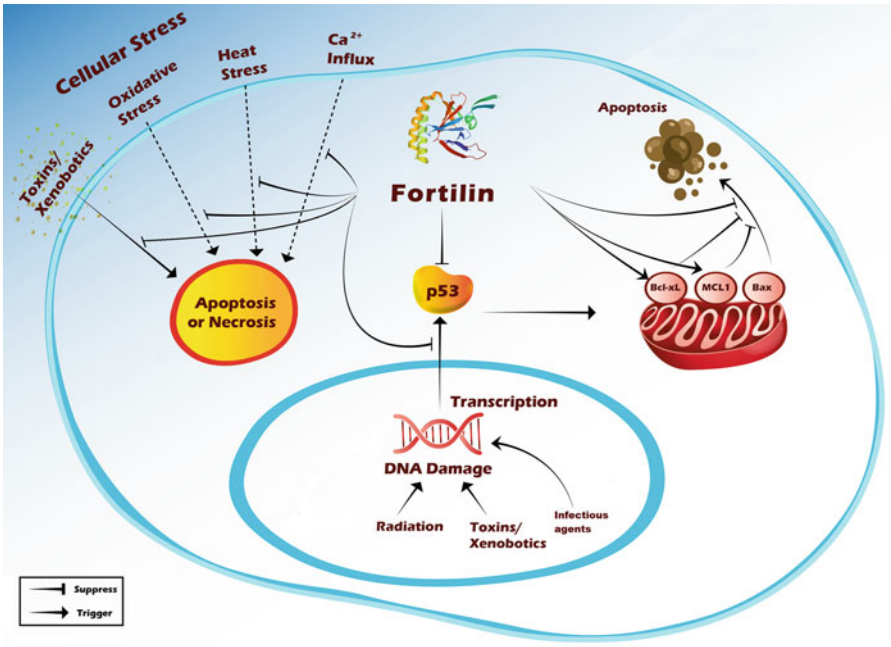

Fig. 4 Fortilin response to general stress. This figure demonstrates the intracellular response of fortilin against different stress conditions such as toxins, heat, and radiation. These pathological conditions trigger either p53-dependent or p53-independent apoptosis, while fortilin acts as anti-apoptotic via interaction with different proteins and other molecules

Fortilin and Sodium-Potassium ATPase

Fortilin inhibits the ionic pump sodium-potassium ATPase (Na, K-ATPase) that serves in signal transduction through regulating cellular membrane potential. This inhibition initiates pathological conditions, such as hypertension and cataract development (Jung et al. 2018). In transgenic mice, fortilin overexpression induced tumorigenesis by signaling and activating non-receptor protein tyrosine kinase (Src)-induced cell proliferation and migration. Figure 5 depicts the interaction of fortilin with the pump Na, K-ATPase.

Fortilin and Calcium Ion

Fortilin possesses the calcium ion (Ca^{2+}) binding ability at the N-terminus (amino acids 1–72) with a dissociation constant (K_d) of 10 μM. Binding of fortilin to Ca^{2+} induces conformational changes that form lower-affinity calcium-binding sites (Graidist et al. 2007). Flow dialysis showed that fortilin has at least two Ca^{2+}-binding pockets that scavenge the rising intracellular Ca^{2+} concentration in response

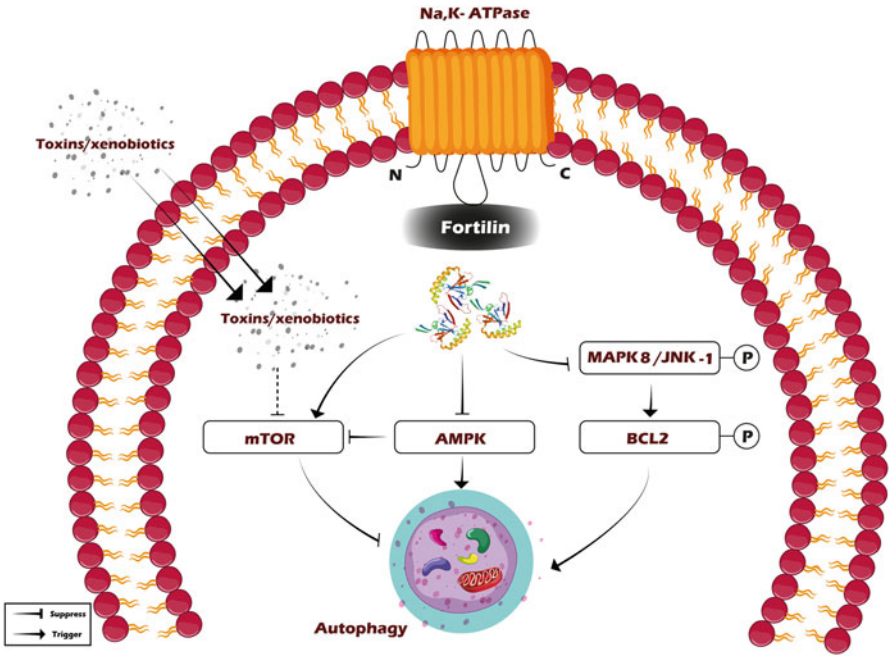

Fig. 5 Fortilin interaction with the Na, K-ATPase pump. This figure illustrates the interaction between fortilin and Na, K-ATPase pump that inhibits autophagy to eliminate dead organelles. The latter is a prerequisite to renew and generate new cellular components. Toxins and xenobiotics could alter cellular signaling and impact this major cell process

to an apoptotic stimuli (Graidist et al. 2007). Such organized steps sequester Ca^{2+} intracellularly and protect cells against Ca^{2+}-dependent apoptosis. The lack of intracellular fortilin significantly elevated intracellular Ca^{2+} (Li et al. 2001). In *Trypanosoma brucei*, the causative agent of African trypanosomiasis, fortilin was also reported to prevent calcium-dependent apoptosis (Li et al. 2001). In fortilin-deficient cells (U2OS cells treated with $siRNA_{fortilin}$), buffering Ca^{2+} with BAPTA-AM (a cell-permeable analog of BAPTA) effectively blocked the ability of thapsigargin (a noncompetitive inhibitor of the sarco/ER Ca^{2+} ATPase) to induce apoptosis by releasing Ca^{2+} from ER-rich microsomes into the cytosol (Graidist et al. 2007). In Jurkat cells (an immortalized line of T lymphocytes), anti-Fas-induced apoptosis raised intracellular Ca^{2+} levels resulting in glucocorticoid-induced apoptosis and DNA degradation (Li et al. 2001). When these cells were challenged, in another study, by anti-human-Fas IgM antibody apoptosis, they started releasing fortilin into the medium before plasma membrane integrity was compromised (Sinthujaroen et al. 2014). Similarly, C57BL/6 J mice challenged with Jo2 anti-Fas (CD95) antibody liver apoptosis demonstrated high fortilin levels in serum (Sinthujaroen et al. 2014).

Fortilin's Role in Apoptosis

Fortilin is essential for the survival of many cancer cells; its overexpression protects cells against apoptosis induced by chemotherapeutic agents such as 5-fluorouracil (5-FU) and etoposide (Fujita et al. 2008). Serum fortilin level was declared more robust and sensitive than previously reported apoptosis markers, such as fragmented cytokeratin-18, cytochrome c, and nucleosomal DNA (Sinthujaroen et al. 2014). Though the relationship between the serum fortilin levels and the extent of apoptosis remains unknown, fortilin is recognized as a novel prosurvival molecule that protects cells against various noxious stimuli including toxins and xenobiotics (Sinthujaroen et al. 2014). Therefore, fortilin depletion would precipitate apoptosis or increase sensitivity to cytotoxic stimuli. Different mechanisms that involve fortilin along with other biomolecules are illustrated in this chapter.

Fortilin and p53-Independent Antiapoptosis

Fortilin was reported as a novel *in vitro* and *in vivo* myeloid cell leukemia sequence 1 (MCL1)-interacting protein, a member of the Bcl-2 antiapoptotic family that is localized predominantly in the nucleus (D Zhang et al. 2002). Fortilin is inducible by serum stimulation and heavy metals and is highly expressed in cancer cell lines making it more resistant to chemotherapy (Graidist et al. 2007). Its overexpression protected HeLa and U2OS cells against apoptosis by blocking the caspase-3-like activity (Li et al. 2001), while its depletion induced spontaneous cell death in MCF-7 (Li et al. 2001) and U937 cells (Graidist et al. 2007). Fortilin was instantly expressed in serum-stimulated human aortic vascular smooth muscle cells (D Zhang et al. 2002). Both proteins are enhancing the intracellular prosurvival conditions because MCL1 is a chaperon of fortilin that stabilizes fortilin (D Zhang et al. 2002). Robust investigation revealed that fortilin is also antiapoptotic in the absence of MCL1. Fortilin functionally antagonizes Bax, a proapoptotic Bcl-2 family member, by inserting itself into the mitochondrial membrane and preventing Bax from dimerizing within the membrane (Susini et al. 2008). Another mechanism by which fortilin protects cells against apoptosis is binding and destabilizing the proapoptotic molecule transforming growth factor-β-stimulated clone-22 (TSC-22) (Chen et al. 2011).

Toxins and xenobiotics induce the production of reactive oxygen species (ROS), which in turn trigger oxidative stress. The antioxidant enzyme peroxiredoxin-1 (PRX1) is abundantly expressed in all cells to scavenge ROS, and in hypoxia, it is transcriptionally activated by Nrf2 (Chattopadhyay et al. 2016). It was found that PRX1-lacking mice exhibited shortened life span due to hemolytic anemia. Fortilin interacts physically with PRX1 to protect it from degradation by proteasomes and blocks its phosphorylation by the serine/threonine kinase Mst1 (Chattopadhyay et al. 2016). Interestingly, fortilin and PRX1 collaboration is involved in the protection of the liver against alcohol-induced injury, the third leading cause of death in the United States (Chattopadhyay et al. 2016).

Fortilin and p53-Dependent Antiapoptosis

Fortilin is upregulated in many human malignancies and binds to the sequence-specific DNA-binding domain of p53, which blocks p53-induced transcriptional activation of Bax (Chen et al. 2011). The most critical defense against tumorigenesis is p53 that becomes powerless through inhibitors and mutations. The antiapoptotic ability of p53 enables it to eliminate cancerous cells through either the transcriptional activation of proapoptotic genes such as Noxa, PUMA4, and Bax or the direct transcription-independent activation of Bax on mitochondria (Chen et al. 2011). Growing cancers mange to keep p53 in check either by mutating p53 gene itself or by expressing p53 inhibitors such as Mdm2. The binding of Mdm2 to p53 results in ubiquitination and proteasome-mediated degradation of p53, making the half-life of p53 very short and intracellular levels of p53 very low (Chen et al. 2011). Mice lacking p53 spontaneously develop numerous neoplasms within 6 months. Furthermore, cell stress such as DNA damage decreases the degree of SUMOylation (a posttranslational modification involved in various cellular processes) of Mdm2 and increases its degradation leading to the stabilization of p53 (Chen et al. 2011). Fortilin also prevents UV-radiation-induced apoptosis, which is entirely dependent on p53 with its signal transducer p85 (Chattopadhyay et al. 2016).

Fortilin as a Biomarker of Toxicity

Up to date, there are few studies that have explored fortilin as a biomarker of toxicity, yet accumulating evidences have demonstrated the effect of different toxins and xenobiotics on fortilin levels *in vivo* and *in vitro*. For example, fortilin was upregulated in the earthworm *Lumbricus rubellus* when exposed to heavy metals including lead, zinc, and cadmium (Li et al. 2001). Fortilin half-life was shortened by the anti-malaria agent dihydroartemisinin (DHA), which also reduced fortilin cellular levels by increasing its ubiquitination in a proteasome-dependent pathway (Fujita et al. 2008). Fortilin demonstrated a protective effect against drug-induced cardiac dysfunction in mice (Cai et al. 2019). Fortilin upregulation in response to heavy metal represented a built-in survival mechanism in other organisms such as *L. rubellus* against noxious environmental conditions, while the covalent modification of fortilin by artemisinin in *Plasmodium falciparum* led to functional inactivation of fortilin and consequent apoptotic death (Li et al. 2001). Fortilin partially protected *Drosophila* Kc cells against the insecticide deltamethrin-induced cell death (Ying et al. 2019). The study proved that this protein plays an important role in the regulation of deltamethrin resistance.

Some studies exposed fortilin as a biomarker of toxicity. For instance, the effect of sublethal polycyclic aromatic hydrocarbons (PAHs) levels in soils was assessed by testing their impact on expression of the reproduction regulating gene annetocin and on the tumorigenic response gene fortilin in the earthworm *Eisenia fetida* cultured in artificial soil (spiked with phenanthrene, pyrene, fluoranthene, or benzo-α-pyrene) (S Zheng et al. 2008). Annetocin and fortilin were both upregulated

by benzo-α-pyrene, while fortilin was downregulated by phenanthrene. Only phenanthrene impacted earthworm weight loss significantly, and no significant differences on cocoon production were observed. The study highlighted the ecotoxicity of sublethal PAHs in soil and proved that fortilin mRNA transcription level in earthworms was more sensitive indicator of PAHs' exposure than traditional indexes, such as cocoon production. In another study, acute stress condition after copper sulfate ($CuSO_4$) treatment was assessed by measuring fortilin gene expression in the Chinese mitten crab *Eriocheir sinensis* (EsTCTP) (Wang et al. 2011). After 8 hours, the EsTCTP expression in hepatopancreas cells increased significantly, peaked at 24 hours, and remained high even after 72 hours. The outcomes demonstrated EsTCTP as an acute-phase protein during copper stress and generally inferred its anti-stress role in invertebrates.

Occupational exposure to bromide (Br^-), which exists naturally in seawater and synthetically as a byproduct of industries (e.g., pesticides and flame retardants), was evaluated in the serum of 397 individuals correlated with genotoxicity markers (DNA damage) and with apoptosis marker fortilin (Nusair et al. 2019). The Br^- levels were significantly correlated with genotoxicity but not apoptosis. The study concluded that the body burden in the selected population provoked genotoxicity but not sufficiently apoptosis, and hence, fortilin could serve as a tool to assess Br^- exposure. Another investigation evaluated methomyl toxicity by measuring fortilin and S100A1 in serum and cardiac tissues of Sprague-Dawley rats (Nusair et al. 2018). Serum fortilin levels increased methomyl exposure, which is reflecting the degree and extent of apoptosis in response to toxicity. Methomyl affected fortilin by reducing Ca^{+2} ATPase activity that alters Ca^{+2} homeostasis (Manawadi and Kallwal 2010). In another word, releasing cellular Ca^{+2} in response to methomyl toxicity triggered further release of fortilin to combat the excess Ca^{+2} fluxes. Methomyl also promotes oxidative stress by enhancing lipid peroxidation, which ultimately induced apoptosis by releasing autolytic lysosomal enzymes and free radicals (Moon et al. 2014). Figure 6 combines the possible intracellular scenarios in response to toxic stress. Taken together, fortilin could be added to the currently available battery of markers for assessing toxicity.

Fortilin Investigation Methods

Multiple methods have been utilized to detect, visualize, and quantify fortilin *in vitro* and *in vivo*. These main methods are summarized in Fig. 7 and are presented herein.

Northern, Southern, and Western Blot

The principle of this method is using DNA to detect RNA (northern blot), using DNA to detect DNA (southern blot), and using antibody to detect protein (western blot). A number of studies have used northern blot to detect fortilin and investigate its different roles. For instance, the role of fortilin and macrophage inhibitory

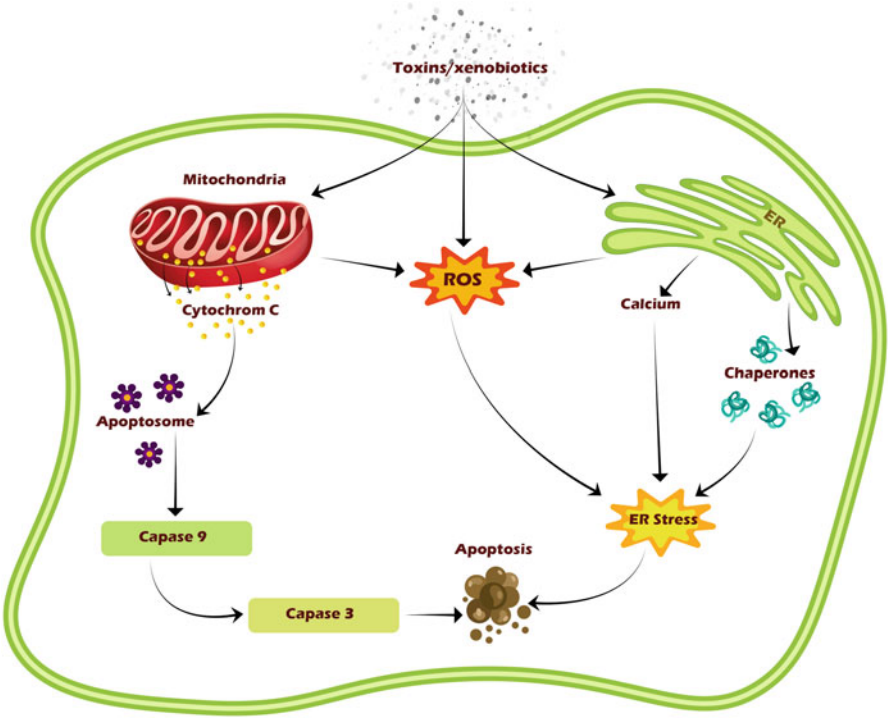

Fig. 6 Mechanisms of toxins for apoptosis induction. This figure demonstrates two pathways (ER stress and caspase) by which different toxins and xenobiotics induce cellular apoptosis

cytokine 1 (MIC-1) in prostate cancer dissemination was investigated using PC-3 prostate cancer epithelial cell line variants and tissues (Patrikainen et al. 2007). The cells were screened for fortilin and MIC-1 gene expression differences during cancer progression by cDNA microarray analysis. Then, the selected genes were further analyzed by northern blot analysis using mRNA isolated from the prostatic cell lines and tissues. The study found that gene of human fortilin and MIC-1 were over-expressed in PC-3 cells.

Since overactivation of the BMP pathway (multifunctional growth factors structurally belong to the TGFβ superfamily) leads to apoptosis during mammalian development and fortilin negatively regulates apoptosis, a targeted disruption of the fortilin gene in mice was performed to study the role of fortilin and BMPs in mammalian development (Koide et al. 2009). Fortilin+/− mice survived and exhibited normal growth, while fortilin−/− mice died around 3.5 days post coitum. Similarly, cultured blastocysts from fortilin+/− embryos exhibited normal outgrowth, while that of fortilin−/− embryos either failed to outgrow or prematurely disintegrated. Northern blotting revealed fortilin overexpression, and the study concluded that inhibition of BMP pathway by fortilin during the early phase of development was necessary for the embryo survival.

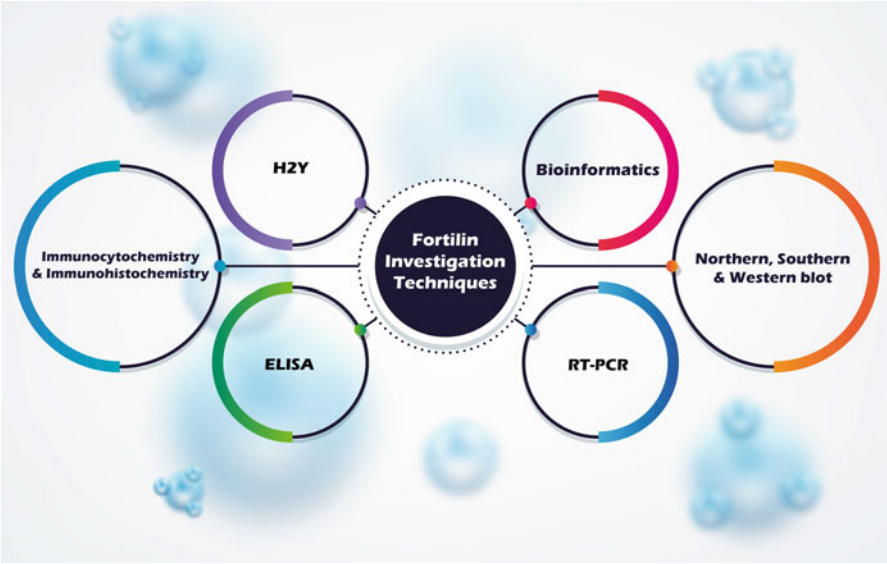

Fig. 7 Fortilin investigation techniques. Infographic demonstration of the main methods used to investigate fortilin including qualitative and quantitative techniques

Southern blot analysis investigated Pm-fortilin role in protecting shrimp from WSSV infection (Tonganunt et al. 2008). Southern blot hybridization of FBP1 DNA with *Penaeus monodon* chromosomal DNA that was digested by a restriction endonuclease enzyme isolated from *E. coli* (EcoRI) responsible for encoding a fortilin-binding protein confirmed fortilin production during the viral infection.

Western blot of total cell lysates followed by chemiluminescence screening and quantification showed that fortilin is ubiquitous in normal tissue, especially liver, kidney and small intestine with more extensive expression in cancer cell lines, such as H1299, MCF-7, and A549 (Li et al. 2001). Another study used western blot that characterized the expression patterns of fortilin in 45 glioma compared to 22 normal brain tissues (Miao et al. 2013). The findings confirmed the overexpression of fortilin and its association with tumor progression in glioma. Western blot analysis was also used to analyze the expression of fortilin in cutaneous Squamous cell carcinoma (SCC) samples using two epidermoid SCC cell lines, A431 and SCL-1, against normal skin keratinocytes HaCaT (Wu et al. 2012). Results significantly associated the increasing fortilin overexpression with the inclining grade of malignancy.

Immunocytochemistry and Immunohistochemistry

Immunohistochemistry is a staining technique of entire sections of tissues for detection and visualization of proteins or other antigens in cells using specific antibodies. These antibodies are linked to a reporter (e.g., fluorophore or enzyme).

Immunocytochemistry, on the other hand, is a staining technique that stains individual layers of cells. After the event of antibody-antigen binding in samples, the enzyme or dye is activated, and the target antigens are examined under microscope. Both staining techniques have been utilized to visualize fortilin for diagnosing cancer and other pathological changes. For example, the application of both immunocytochemistry on HeLa cells transiently expressing FLAG-tagged fortilin as well as immunohistochemistry on human breast ductal carcinoma tissue confirmed that fortilin is predominantly localized in the nucleus (Li et al. 2001). In addition, immunohistochemistry confirmed the binding site between Pm-fortilin and FBP1 during WSSV infection by co-localization of both proteins in cells (Panrat et al. 2012).

Yeast Two-Hybrid Assay (Y2H)

Yeast two-hybrid system (Y2H), also known as two-hybrid screening, is a technique discovering actual physical interactions of protein to protein as well as protein to DNA. Previous studies performed Y2H to investigate fortilin. For example, the binding between fortilin and MCL1 was confirmed using Y2H, which quantified hydrophilic scores representing the actual physical interactions between fortilin and MCL1 (Li et al. 2001). Human fetus liver library was screened by Y2H library, and the bait was full-length MCL1 cloned into pAS2.1 (a vector encodes GAL4 DNA-binding domain). Another study screened yeast to identify rat fortilin-binding proteins; using Y2H, rat skeletal muscle cDNA library was screened (Yoon et al. 2000). The bait protein was a 48 kDa protein. The amino acid sequences were analyzed by ANTHEPROT computer software.

Enzyme-Linked Immunosorbent Assay (ELISA)

Fortilin concentration was measured in the serum samples from methomyl-intoxicated rats (Nusair et al. 2018) and from bromide-exposed individuals (Nusair et al. 2019) using enzyme-linked immunosorbent assay (ELISA). Anti-fortilin antibodies were used, and the optical density of samples was measured by a spectrophotometer. Another study used ELISA to prove that fortilin was upregulated at the translational level in the infective-stage larvae of *Trichinella pseudospiralis* (Mak et al. 2007). The study suggested that fortilin production was necessary for the process of heat-stress adaptation in nematodes.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis

RT-PCR is a laboratory method combining reverse transcription of RNA into complementary DNA (cDNA) and amplification of selected DNA segments using PCR (Mak et al. 2007). The role of fortilin mRNA and protein expression as a

prognostic tool was investigated in different tumor types. For example, RT-PCR analysis of the survival rate of 5651 cancer patients significantly associated high fortilin mRNA expression with decreasing survival rate in lung, prostate, breast, and ovarian cancers (Fischer et al. 2021). Fortilin mRNA expression proved fortilin's importance for rumen epithelium development in Hu sheep (K Zheng et al. 2021). The study also reported that AKT phosphorylation regulated the translation of fortilin mRNA. Another study explored the function and regulation mechanism of fortilin mRNA in hepatocellular carcinoma (HCC) using mRNA transcription level (Liu et al. 2020). Fortilin was overexpressed in HCC patients. However, fortilin knockout HCC cells exhibited attenuated proliferation, migration, and invasion. The knockdown of fortilin by siRNA effectively reduced fortilin mRNA levels but not protein levels in HCC cells. Findings indicated that fortilin mRNA and fortilin protein formed a regulatory circuit to keep the homeostasis of cellular fortilin levels (Liu et al. 2020).

Recommendations

Fortilin could be further exploited in a noninvasive diagnostic tool to determine the severity of toxicity cases and to reveal additional regulatory mechanisms of apoptosis, human tumorigenesis, and other proliferative diseases. Further investigations are recommended to validate the potential clinical application of fortilin to protect against toxicities such as using fortilin-PRX1 interaction to protect against alcohol-induced liver damage. Micro-RNAs and small molecules could be used to enhance fortilin expression in hepatocytes to protect the liver against ROS-mediated damage induced by acetaminophen overdose and ischemia-reperfusion injury following liver transplantation.

Applications to Prognosis

In this chapter, there are different applications of fortilin in the current and future scientific and medical sectors. For example, since fortilin is actively secreted before destruction of the cellular plasma membrane, it would form an excellent prognostic biomarker for in vivo as well as in vitro toxicity cases. Fortilin also could have the ability to readily assess the degree of toxicity in human through the association between its expression levels and the degree of toxicity. Furthermore, fortilin serum levels highly increase after apoptosis-inducing therapy; therefore, it would be used as a tool to monitor the patient response to different therapeutic agents. Fortilin has also been discussed as a prognostic factor for prognosis of diseases, cancer, and other pathological conditions. Thus, evaluations of large numbers of different cancer types could be performed by screening fortilin mRNA transcription levels using quantitative RT-PCR analysis (Fischer et al. 2021; Mak et al. 2007). Considering that fortilin serves in cell-cell communication, it would convey the toxicity massage from

intoxicated cells to blood, for instance. Therefore, it is promising to consider such protein in the toxicity biomarker panel.

Bearing in mind the implications of human fortilin, similarly, recombinant fortilin (rFortilin) also has reported implications. For example, the recombinant fortilin from Fenneropenaeus merguiensis (rFm-Fortilin (FL)) demonstrated protective and proliferative activity on human dental pulp cells against 2-hydroxy-ethyl methacrylate (HEMA) toxicity from filling cement to pulp cells (Kedjarune-Leggat et al. 2020). The analog promoted growth and mineralization by binding the surface of Rab GTPase and suppressed production of proinflammatory cytokines, including histamine and TNF-α. The study declared that rFm-Fortilin (FL) has potential use as a supplementary medical material to promote cell proliferation in patients suffering severe tooth decay.

Applications to Other Diseases or Conditions

In this chapter, the discovery of physical and functional interaction between fortilin and p53 has significant clinical implications. First, antagonizing this interaction may reactivate p53 and induce apoptosis within cancer cells that harbor wild-type p53; despite that, this strategy does not work in cancers that harbor a mutated p53 (Chen et al. 2011). Using fortilin small molecules to antagonize p53 would improve the effectiveness of cancer therapeutic agents such as radiation, chemotherapy, Nutlin-3A, and MI-219 (small molecules that disrupt the p53-Mdm2 interaction). Furthermore, this antagonism also may have applications in the treatments of atherosclerosis refractory to statins because the lack of functioning macrophage p53 is associated with accelerated atherosclerosis (Chen et al. 2011).

Fortilin has been linked to a number of fundamental biological processes, including tumorigenesis and cancer progression. Fortilin gene expression could be implemented in the development of anticancer therapy (Fischer et al. 2021). For example, the role of fortilin in skin carcinogenesis was investigated by silencing the TPT1 gene using siRNA in the cutaneous SCC cell line A431. The expression of fortilin in cell line was significantly downregulated by transfection with fortilin siRNA. The downregulation decreased cell proliferation and increased apoptosis suggesting that the TPT1 gene could be a potential therapeutic target in skin SCC (Wu et al. 2012). Additionally, rFm-Fortilin was found to have the potential use as a supplementary medical material to promote cell proliferation in patients suffering severe tooth decay and other tooth conditions [9]. Injection of rFortilin into spot syndrome virus (WSSV)-infected shrimp resulted in 80–100% survival rate (Tonganunt et al. 2008). The study showed that fortilin, among other molecules such as eIF5A and 14-3-3, participated in signal transduction during viral replication and decreased viral infection through yet unknown mechanism. Such findings encourage future applications of rFortilin in the development of prophylaxis and therapeutic agents for diseases as well as toxicities.

Mini-dictionary of Terms

- **Fortilin**: A multifunctional protein translationally controlled tumor protein (TCTP), also called histamine releasing factor (HRF), that presents in most eukaryotic organisms. Its main function is antiapoptotic protein.
- **Ehrlich ascites carcinoma (EAC)**. A murine mammary adenocarcinoma model where fortilin protein was first discovered.
- **Unfolded protein response (UPR)**. An endoplasmic reticulum (ER)-stress defense program in which fortilin plays an important role against apoptosis and cells death.
- **Anti-Fas-induced apoptosis**. An agonistic anti-Fas antibodies that induce apoptosis of cells expressing Fas on the membrane by triggering a cascade of caspases.
- **SUMOylation**. A posttranslational modification involved in various cellular processes.
- **Proteasome-dependent pathway**. A pathway that degrades proteins by proteolysis. For degradation to occur, proteins are tagged with a small protein called ubiquitin. Ubiquitin ligase enzymes catalyze this tagging reaction.

Key Facts of Fortilin and Fortilin Recombinant

It is a hydrophilic protein of 18–23 kDa.

It has no sequence similarity to any other previously sequenced proteins.

It is characterized by the selective reduction in the diameter of type II fibers.

Fortilin exists in various normal tissues with much higher levels in cancer cell lines.

It was first described as a growth factor–induced protein in murine cell lines.

Its response upon noxious stimuli is either upregulated or downregulated or structurally modified.

Recombinant fortilin has reported medical and scientific implications with no disease complications.

Summary Points

- Fortilin is the multifunctional protein translationally controlled tumor protein (TCTP).
- Cellular fortilin levels are highly regulated by variety of biological signals and molecules.
- Fortilin transcripts accumulate in resting cells and are rapidly translated into the protein on the basis of cells' requirements.
- Fortilin is involved in protein synthesis by binding to the elongation factors of the EF1 family with other translational apparatus.

- Fortilin binds to p53-MDM2 complexes and inhibits MDM2 auto-ubiquitination, resulting in MDM2-mediated ubiquitination and degradation of the protein p53.
- There are few studies that have explored fortilin as a biomarker of toxicity, yet accumulating evidences have demonstrated the effect of toxic exposure to different substances and xenobiotics on fortilin levels in vivo and in vitro.
- Multiple methods have been utilized to qualify and quantify fortilin in vitro and in vivo, for example, immunoblotting, immunostaining, enzyme-linked immunosorbent assay, yeast two-hybrid system, and reverse transcriptase polymerase chain reaction.

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Antibodies as Biomarkers: Effect of Microcystin Exposure

5

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Abstract

Microcystins are toxins produced by cyanobacteria of the genus *Microcystis*. When cyanobacteria proliferate in fresh or brackish waters, microcystins become a risk for public health, particularly in water bodies used as a drinking supply and for recreation. *Microcystis* spp. and microcystins (MCs) are globally distributed, and under climate change conditions, this phenomenon is expected to increase, and so is the health risk. MCs are mainly hepatotoxic; the intoxication is difficult to diagnose because its symptoms are nonspecific and easily confused with those of other diseases. Although there is vast evidence of the health problems associated with this exposure, there are no simple and specific biomarkers available to help evaluate the epidemiological impact and the extent of human exposure or even to diagnose the intoxication. This chapter reviews the toxicity mechanisms of MCs and the biochemical parameters altered when exposed to MCs. The value of specific antibodies as potential biomarkers is also discussed.

Keywords

Algal blooms · Cyanobacteria · *Microcystis* spp. · Cyanotoxins · Microcystins · Microcystin-LR · Intoxication · Antibodies · Exposure biomarker · Chronic exposure · Acute exposure · Hepatotoxicity

Abbreviations

Abs	Antibodies
ADDA	3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
ADME	Absorption, distribution, metabolism, and excretion
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AST	Aspartate transaminase
ATCC	American type culture collection
CaMKII	Calcium-calmodulin-dependent protein kinase II
ERK	Extracellular signal-regulated kinases
GGT	Gamma glutamyltransferase
GRP94	Glucose-regulated protein 94
GSH	Reduced glutathione
HL7702	Normal human hepatic cell line
Igs	Immunoglobulins
JNK/SAPK	Mammalian stress-activated protein kinase
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinases
MC	Microcystin
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MC-LR	Microcystin-LR

MCs	Microcystins
MCV	Mean corpuscular volume
OATPs	Organic anion transporting polypeptides
p38	Mammalian stress-activated protein kinase
PLT	Platelets
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
RBC	Red blood cells
SOD	Superoxide dismutase
WBC	White blood cells

Introduction: Cyanobacteria, Their Toxins, and Health Risk

In recent decades, the excessive growth of cyanobacteria in different water bodies (rivers, lakes, reservoirs, and estuaries) has become an increasingly relevant sanitary problem (Carmichael and Boyer 2016; Cheung et al. 2013; Reichwaldt and Ghadouani 2012), which is expected to become even worse during the next years as a consequence of a changing climate and the environmental crisis (Michalak et al. 2013; Paerl and Huisman 2008). Cyanobacteria are also known as blue-green algae, and they are part of the phytoplankton community in the aquatic ecosystems. These bacteria are ancient microscopic organisms that can survive in fresh waters, in brackish waters, and even in soil – either on the surface or beyond it – according to their strain. When they reproduce excessively in aquatic environments, they form bodies of floating cyanobacteria, a phenomenon known as cyanobacterial blooms. This phenomenon usually results from a high input of nutrients like phosphorus (P) and nitrogen (N) due to human activity – a process commonly named eutrophication – and to a temperature higher than 15 °C (Gilbert 2017). For these reasons, blooms are more frequent during the summer. When they occur, organic matter from phytoplankton biomass floats in the water surface conferring a specific odor and an intense blue-green color (WHO 2015).

Some cyanobacterial strains, though not all of them, have the ability to produce toxins. These toxins can be classified based on their chemical structure, their toxicity effect, and their origin. They are generally known as cyanotoxins. The most frequent are anatoxins, nodularins, and microcystins, which were originally named after the cyanobacteria genus that was first observed to produce them (*Anabaena*, *Nodularia*, *Microcystis*, respectively). Microcystins (MCs) – one of the most abundant in water bodies – share among them (and with nodularins) a non-proteogenic aminoacidic group named ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) (Carmichael 1992). Up to date, more than 250 congeners of microcystins have been characterized, with molecular weights that range between 800 and 1100 Da (Fastner and Humpage 2021).

The ability of cyanobacteria to produce toxins makes blooms a general health problem, and this fact has been known for more than 200 years (Francis 1878). Ample evidence indicates that there is a proven risk when contaminated waters are

consumed and also during recreational activities that involve contact with it (Backer et al. 2010). This risk has been documented for numerous animal species besides human beings, like sheep, cattle, horses, pigs, dogs, fish, rodents, amphibians, ducks, bats, flamingoes, zebras, and rhinoceros (Valerio et al. 2010). Their relevance for human health is so well known that the WHO considered this as an emerging issue in water (WHO 2003) and has provided water quality guidelines for testing cyanotoxins in drinking and recreational waters (WHO 2020). Nevertheless, there is no established method yet for diagnosing intoxication with cyanotoxins (Su et al. 2020).

Toxic algal blooms are a worldwide problem, and South America is not exempt from them; yet information concerning algal blooms in this region is still scarce (Svirčev et al. 2019). In Argentina, an increasing number of episodes have been documented since 1990, probably in association with changes in human activities, like urbanization, the introduction of new agricultural practices, and the discharge of organic matter into water bodies without the corresponding prior treatment. Although the negative consequences for health and for the environment are evident, regulation in Argentina is far behind other countries in the region, like Uruguay and Brazil, which have, for example, incorporated cyanotoxin surveillance in recreational water and water for consumption as a routine practice (Aguilera et al. 2018). Brazil has a long history of algal blooms with cyanobacteria. According to some authors, the frequency of these blooms justifies the implementation of surveillance strategies on water reservoirs, considering their high toxicity for humans and mammals in general (Bittencourt-Oliveira et al. 2014). The problem also affects other countries from the Southern Cone, like Uruguay, which revised the incidence of cyanobacterial blooms and contamination of water bodies in research papers from 2015 (Bonilla et al. 2015) and 2020 (Juanena et al. 2020), and Chile (Campos et al. 2005), which has studied algal blooms in continental water bodies as a part of an approach to a problem that has been affecting them with increasing concern over the last three decades.

According to reports from many parts of the globe, it is commonly accepted that intoxication with cyanotoxins might occur through skin contact (dermal exposure route, when contaminated waters come into contact with skin, e.g., during recreational activities), through the respiratory tract (inhalation route, when water enters via inhalation during recreational activities), through the digestive tract (oral route, when contaminated water or food is ingested), and other more rare situations like during dialysis treatment (intravenous route) (Poste et al. 2011; USEPA 2015). Depending on the exposure route, the exposure time, the magnitude of the bloom, and the ability of cyanobacteria to produce toxins, the result can be an acute or a chronic clinical condition. In some extreme cases, the intoxication can be lethal.

Although not frequent, there are several reports concerning clinical symptoms due to acute exposure to microcystins. An excellent revision regarding this subject is the one published by Wood (2016), in which the authors suggest that the lethal dose (LD_{50}) of MC-LR is about ten times lower than that of strychnine and 200 times lower than sodium cyanide. Besides, the authors point out that the relation between toxicity and dose has a sharp curve with an abrupt slope; thus, when toxic effects

appear, the dose is already close to being lethal. In that revision, acute cases are described from 1930 until the present day, including a wide spectrum of circumstances, ranging from recreational exposure to contamination during medical procedures and occupational contact (Wood 2016). In general, it is considered that an acute clinical condition can be mild, moderate, or critical, and it is characterized by gastrointestinal symptoms (nausea, vomiting, fever, headache), a respiratory syndrome (asthma, pneumonia, cold symptoms), allergic reactions (rash, dermatitis, skin peeling), and hepatic toxicity, which can be followed by multiple organic failure and death (Pérez et al. 2008; Testai et al. 2016).

On the other hand, the available evidence regarding chronic exposure to toxic algal blooms and its impact on health is overwhelming. Moreover, the number of chronic cases may be underestimated due to both a deficient sanitary control of water quality and the poor knowledge medical practitioners have of this type of intoxication. This underestimation may be exacerbated by the many factors that remain unknown on this subject and by the lack of clear indicators to be used in the diagnostic algorithm of toxicity by cyanotoxins. Concerning these issues, Funari and Testai (2008) indicated that the sanitary risks associated with chronic exposure to cyanotoxins deserve to be considered critical, far beyond the risks associated with acute exposure. In agreement with this concern, the American Academy of Pediatrics (AAP) published a warning document in 2015 regarding the risks associated with the chronic consumption of water contaminated with cyanobacteria, particularly for infants and children (Etzel 2015).

In this review, we have focused on the molecular mechanisms of MCs' toxicity, the effects of chronic exposure to microcystins, and the biochemical indicators that have been used so far to identify intoxication. As the current status of knowledge on specific and easily measurable biochemical indicators used as biomarkers is still insufficient, we here introduce some new insights into the potential use of antibodies as specific and straightforward biomarkers of exposure and for their potential use in routine analyses.

Microcystins: Structure and Toxicity Mechanisms

Chemical Structure

As we have mentioned before, microcystins are the most studied toxins produced by cyanobacteria and the most frequently found in algal blooms. These molecules are cyclic heptapeptides composed of five non-proteogenic amino acids and two additional L-amino acids, which vary from molecule to molecule and are responsible for more than 250 microcystin variants. Nevertheless, the most frequent microcystin is MC-LR, which contains leucine and arginine. Figure 1 shows a general structure for MCs (Fastener and Humpage 2021).

According to the literature, residues 5 and 6 in Fig. 1 (ADDA group and D-glutamic acid) are responsible for the ability of MCs to bind and inhibit phosphatases, as described in the following section, although it has been stated that residues

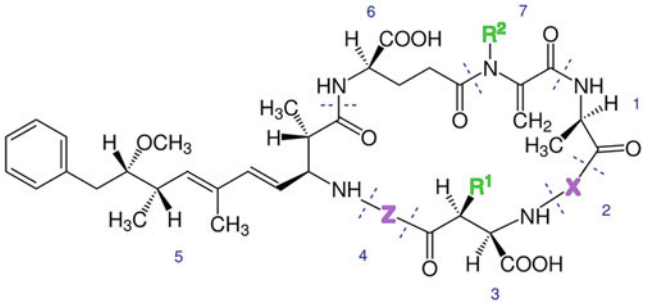

Fig. 1 General structure of microcystins. In the structure, X and Z (violet, positions 2 and 4, respectively) are the variable L-amino acids (in MC-LR, X stands for L-leucine and Z stands for L-arginine); R1 and R2 can be either H or CH3 (green); the rest of the residues are (1) D-alanine, (3) D-erythro-β-methylaspartic acid, (5) ADDA group, (6) D-glutamic acid, (7) N-methyl-dehydroalanine

X and Z confer different hydrophilicity profile to the molecule, and this has been linked to toxicity levels in animal models (McLellan and Manderville 2017; Vesterkvist et al. 2012).

The ADDA group is present in most MCs, as well as nodularins. This chemical group has been used to raise antibodies against these toxins in the development of the commercial enzyme-linked immunosorbent assays (ELISA) used for the detection of microcystins – and nodularins – in water (Fischer et al. 2001).

It is well known that contact with cyanobacteria cells may cause itchy rashes, eye irritation, and other hay fever-like upper respiratory symptoms; however, it is not clear whether these effects are due to the whole microcystin molecule or just a part of it. Moreover, a recent work has hypothesized that MCs could inhibit or reduce the allergic reactions caused by contact with other more allergenic peptides of the cyanobacteria cells (Geh et al. 2016).

Regarding MCs' metabolism, the group in position 7 (opposite ADDA) has been characterized as being involved in the GSH conjugation for toxin excretion (Testai et al. 2016).

Molecular Mechanisms of Toxicity

Evidence concerning the absorption dynamics, the metabolism, and the mechanism of action of microcystins (ADME) derives from experiments performed in animal models (in vivo assays) and conducted on established cell lines (in vitro assays). So far, there are very few epidemiological reports, probably due to the lack of specific biomarkers for microcystin exposure.

Clinical features associated with chronic exposure to microcystins are closely related to the exposure route and metabolism. After being absorbed and incorporated into the bloodstream, these toxins enter any cell, expressing any of the organic anion transporting polypeptides (abbreviated as OATPs) on its surface (Fischer et al. 2005;

Chen et al. 2016), including cells from the kidneys, brain, reproductive organs, and liver, the latter being one of the most widely affected organs, according to the specific literature.

From an experimental perspective, Heinze (1999) explored the effects of chronic intake of contaminated water during 28 days in a murine animal model, using two different doses of MC-LR (50 μg/kg and 150 μg/kg), and demonstrated that in these conditions several hepatic enzymes (ALP and LDH) are altered, as well as the general weight of each animal and the weight of their liver. Also, livers from affected animals had pronounced hemorrhagic necrosis and a considerable accumulation of Kupffer cells. These results have been reinforced by several research groups who analyzed the utility of established cell lines to gain knowledge on the toxicity mechanism of microcystins. For example, Menezes' group (Menezes et al. 2013a) demonstrated the utility of two hepatocyte cell lines – the AML12 (hepatocytes from *Mus musculus*, ATCC-CRL 2254) and the HepG2 cell line (human hepatoma, ATCC-CRL 10741) – and a renal cell line, the Vero-E6 (kidney epithelial cells from the African green monkey, *Cercopithecus aethiops*, ATCC-CRL 1586). In those experiments, the authors found a dose-dependent toxic effect, which consists of a reduction of cell viability, induction of apoptosis, activation of autophagy mechanisms, relocalization of GPR94 – an indicator of stress of the rough endoplasmic reticulum – and the disruption of lysosomes and mitochondria (Menezes et al. 2013b).

All the specific literature agrees that the fundamental mechanism – though not the only one – of microcystin toxicity depends on its ability to inhibit serine and threonine phosphatases PP1 and PP2A (Carmichael 1994). This inhibition leads to hyperphosphorylation of many regulatory proteins that are key to signaling transduction and intracellular metabolism. Considering that these phosphatases are involved in numerous intracellular metabolic routes, the wide range of processes that are affected by microcystins should come as no surprise, nor should the many organs that are compromised during intoxication thereby. Moreover, this pleiotropism is also explained by the ubiquity of OATPs, which is illustrated further in this chapter.

Alterations of Cell Cycle

In terms of the molecular mechanisms of toxicity, McLellan and Manderville (2017) indicated that the inhibition of PP1 and PP2A alters the regulation of the mitogen-activated protein kinase family (MAPK, which includes p38, JNK/SAPK, and ERK). Their activity is related to cell functions like proliferation, differentiation, mitosis, cell survival, and apoptosis.

The authors also suggest that the inhibition of PP2A and alteration of MAPK routes might lead to the disruption of cell cytoskeleton, as others have demonstrated in a human hepatocyte cell line (HL7702) (Sun et al. 2011), and might be the mechanism for the reported oncogenic effect of microcystins.

According to Campos and Vasconcelos (2010), the oncogenic effect of microcystins mentioned by McLellan might be accounted for by the inhibition of PP1 and PP2A, which reduces the activity of DNA repair systems and activates the calcium-

calmodulin-dependent protein kinase II (CaMKII), a protein known to promote cell apoptosis. In their paper, the authors point out that those effects take place indirectly through the alteration of MAPK – modifying its ability to regulate the expression of several proto-oncogenes related to transcription, cell growth, and differentiation – as well as directly through the alteration of proteins related to the cell cycle, like p53, Bcl-2, and Nek2. For example, it is known that the holoenzyme PP1 binds to Nek2, impairing its role as a regulatory protein kinase of the cell cycle, regarding the progress of phase M and during chromosome segregation at the cell division stage, and that PP2A directly inhibits the activity of p53 through the dephosphorylation of its residue threonine-55 (Li et al. 2007). The p53 protein has a central role as a transcriptional transactivator during DNA repair, apoptosis, and tumor-suppressor pathways. PP2A also inhibits Bcl-2, a protein with antiapoptotic activity. In this context, inhibition of PP1 and PP2A would lead to a lack of control of the cell cycle, which might in turn lead to uncontrolled gene expression, proliferation, and cell differentiation.

Toxicity on Organs

Regarding the toxicity on organs, the paper of McLellan points out that – besides the effect of microcystins on PP1 and PP2A – microcystins also react directly with thiol groups, a fact that has been known for years to be related to hepatic toxicity, as in the case of acetaminophen toxicity. On the other hand, several authors indicate that the damage reaches organs other than the liver, like the kidneys, heart, and reproductive organs, matching the known distribution of OATPs (Chen et al. 2016; Milutinović et al. 2006; Roth et al. 2012; Wang et al. 2008).

The distribution of OATPs might help to explain that 50–70% of the MC-LR, when administered intravenously in animal models, is found in the liver and only 1–5% in kidneys and less than 1% in other organs. After the first few hours, only 15% of the toxin is eliminated through feces, and 9% can be found in urine (Robinson et al. 1991).

Several reports in recent decades have been published regarding cases of toxicity or allergenicity by algal blooms, like the work by Cohen and Reif (1953), who described the case of allergic dermatitis to phycocyanin in the case of a 6-year-old girl suffering from skin rash after bathing in a lake.

In Vivo Studies

Concerning in vivo studies, attention should be given to the research by Milutinović, who characterized the renal toxicity of cyanotoxins in a rat model, after repeated sublethal i.p. doses, demonstrating the existence of histological alterations and cytoskeletal disruption (Milutinović et al. 2002, 2003), and the paper by Nobre et al. (1999), who proved the alteration of renal function in a rat model of isolated kidney after the administration of cyanotoxins in doses comparable to an acute intoxication. Other authors explored the effects of MC-LR on a diverse range of organs, tissues, and doses, like Botha – who investigated the effect of a 75% LD50 on different portions of the gastrointestinal tract in a murine model,

showing the increase in the apoptosis rate in the duodenum, jejunum, and ileum in mice (Botha et al. 2004). Concerning this tract, Kubickova et al. (2019) published a review in which they point out different toxic effects of cyanotoxins that were further confirmed by other authors, like the erosion of the small intestine in mice (Ito et al. 2000) and the disruption of cell cohesion in fish (Djediat et al. 2010). In 2003, Shen et al. analyzed the effects of several doses of cyanotoxins on the phagocytic and proliferative functions of the immune system cells in mice, and in 2016, Lone et al. proved the alteration of hematological parameters of mice exposed to microcystins, like leukopenia, thrombocytopenia, and the decrease in the red cell count, hematocrit, mean corpuscular hemoglobin, mean corpuscular volume, and mean corpuscular hemoglobin concentration (MCH, MCV, and MCHC, respectively). Regarding the incidence of neoplasia, Svirčev et al. (2009) and Hernández et al. (2009) analyzed the prevalence of liver and colorectal cancer in human beings from an epidemiological perspective, and their statistical association with chronic exposure to water with algal blooms in Serbia and China, and found a positive and worrying correlation.

In Vitro Studies

In vitro, as stated in the section concerning molecular toxicity, the ability of microcystins to produce cytotoxicity has been extensively documented for diverse cell lines, like adenocarcinoma (human CaCo-2), astrocytoma (IPDDC-A2), and lymphoblastic cell lines (NCNC), in which an increase has been detected in the production of free radicals of oxygen and damage to DNA (Zegura et al. 2008). The ability of MC-LR to reduce general cell viability has also been demonstrated for Vero-E6 (Diaz et al. 2009), among others.

Biomarkers

Definition and Applications

Considering the abundant and growing scientific evidence about the damage caused by chronic exposure to microcystins, and contemplating the diversity of direct and indirect effects that these toxins have on cells and organs, there is a justified need to find strategies to diagnose the exposure to these toxins, based on reliable indicators.

There are several definitions of biomarkers, which have been discussed elsewhere (Strimbu and Travel 2010). In the field of toxicology, these can be classified in several subgroups according to their use, although a single biomarker can be included in many subgroups. These groups are *exposure* biomarkers, when used to confirm or detect contact with any external agent, substance, or factor; *effect* biomarkers, which include any that can be used to evaluate the response of the organism to a medication, treatment, or external agent; and *susceptibility* biomarkers, when used to evaluate the tendency and/or the susceptibility of the organism to a given factor (Gupta 2014).

Biomarkers of Microcystin Exposure

In studying the effects of microcystin exposure and considering the classification mentioned above, one of the most important challenges is to identify biomarkers that make it possible to confirm the exposure to this group of toxins and to diagnose their toxic effects. So far, several biochemical parameters have been proposed, like hepatic enzymes and metabolites (AST, ALT, FAL, GGT, bilirubin) (Li et al. 2011; Zhao et al. 2020), renal parameters (urea, creatinine), and hematological parameters (WBC, PLT, RBC, hematocrit, MCH, MCV, MCHC), as well as the direct detection of the toxin or its metabolites in the bloodstream. In terms of observable signs, it has also been proposed that cutaneous manifestations, allergic symptoms, and acute respiratory manifestations might be markers of intoxication with microcystins.

While biochemical parameters and observable signs are unspecific and not exclusively linked to microcystin exposure (van der Merwe 2014; Chorus and Testai 2021), thus impairing their use as specific exposure or effect biomarkers, direct toxin detection requires specific equipment and laboratory conditions (Chen et al. 2009; Heussner et al. 2014), and its sensitivity is conditioned by the speed at which the toxin is cleared from the bloodstream and rapidly distributed to the liver, kidneys, and other organs, or eliminated by feces and urine. As an example of the complexity of the situation, Sedan et al. (2013) showed the modification of biochemical parameters in a murine model under chronic exposure to MC-LR; the authors observed alterations in the level of ALT, albumin, bilirubin, methemoglobin, glutathione, and α-tocopherol, together with modifications in the production of free radicals of oxygen, the SOD activity, and the lipidic profile. Nevertheless, the only binding parameter these authors found with MC-LR exposure was the detection of MC-LR in the bloodstream.

Taken together, all this evidence reinforces the need for proper biomarkers, which would allow for adequate diagnosis and propitiate proper epidemiological surveillance and population studies.

Antibodies as Biomarkers

Immunoglobulins (Igs) are proteins produced by activated B lymphocytes (aBL). They are heterotetramers composed of four polypeptides: two heavy chains (H) and two light chains (L). The antigen-binding site – called paratope – is composed of peptide loops belonging to both H and L chains, and as these chains are identical, each Ig has two paratopes (see Fig. 2). Each aBL produces Igs with a single specificity, although the constant regions that determine the isotype can vary in a process named *isotype switch* (see the next section). Once the switch has occurred, it cannot be reversed (Chiu et al. 2019; Schroeder and Cavacini 2010).

Knowing the antigen under study, the presence of specific Igs can be studied in different biological fluids, and detecting it provides information regarding whether the organism has been in contact with that antigen before. Besides the participation of specific Igs – called antibodies (Abs) – in the immune response to that antigen, the

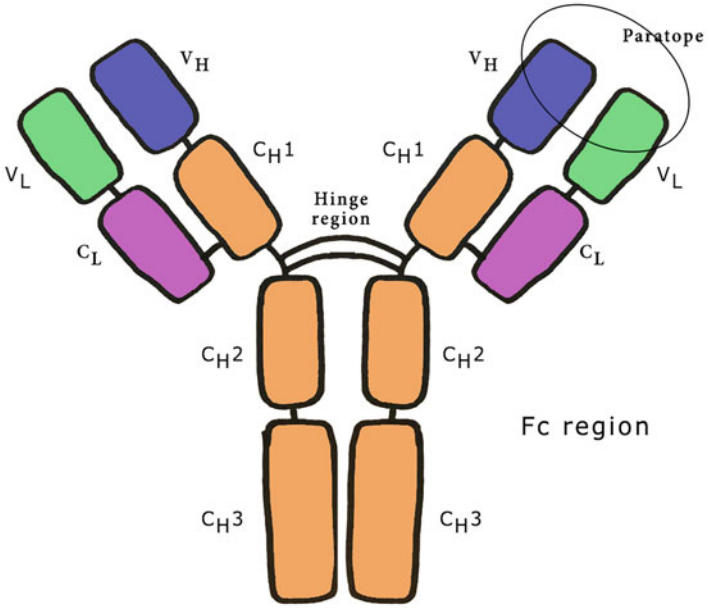

Fig. 2 General structure of immunoglobulins. VH and VL are variable domains of heavy and light chains; CH1, CH2, and CH3 are the constant domains of the heavy chain; and Fc is the name of a region composed of the CH3 domain and part of the CH2 domain

detection of specific Abs can be considered an indicator of the immune response and thus a marker of a prior contact with the antigen.

Specific Abs have been extensively used as biomarkers, as in autoimmune diseases, oncological processes, and infectious diseases. Autoantibodies, which are Igs that recognize self-structures as a consequence of the loss of self-tolerance, are present both in classic autoimmune diseases like lupus or rheumatoid arthritis, conditions in which Abs are also responsible for the clinical symptoms, and cancer, cardiovascular, and neurodegenerative diseases (Aziz et al. 2018).

Fässler et al. (2019) demonstrated that Abs associated to melanoma – IgG specific for tyrosinase-related protein types 1 and 2, glycoprotein 100 (gp100), MelanA/MART1, and the testicular antigen NY-ESO-1 – are good markers of the response of the organism to the treatment with cell-cycle checkpoint inhibitors. With a similar logic, Tikhonov et al. (2020) pointed out that a specific profile of Abs is raised due to abnormal glycosylation, which in turn makes it possible to classify the tumor.

Moreover, Yanamandra et al. (2011) proposed specific Abs to α-synuclein as diagnostic biomarkers for diseases that are characterized by neurodegeneration, like Parkinson's disease, while Srivastava et al. (2012) pointed out the presence of antibodies to potassium channel KIR4.1 in almost 47% of the patients with multiple sclerosis.

The utility of Abs as diagnostic biomarkers in the field of infectious diseases is historical and goes beyond this revision. It has been extensively documented for

viral, bacterial, and parasitic pathologies like HIV, hepatitis, syphilis, and Chagas disease, in which it can be used to distinguish acute from chronic conditions, taking advantage of the isotype switch of specific antibodies raised against the infectious agents.

Concerning Abs as biomarkers for intoxication with natural toxins, there are few reports describing their use and performance in diagnosis (Khojasteh et al. 2003; Lefebvre et al. 2012). The reasons for this lack of information might be that most of the toxins lead to acute conditions in which the adaptive immune system has not enough time to produce specific antibodies.

Isotype Switch of Antibodies

The immune response can be classified as primary, when the antigen first enters the organism, and secondary, when the immune system interacts with it in any further encounter. During a primary Ab response, the dominating antibody isotype is IgM; this Ig has a pentameric structure composed of relatively low-affinity subunits. Because of its large size, IgM can be found mainly in the bloodstream and can rapidly bind antigens and activate the complement system. Microbial carbohydrates mostly lead to the production of specific IgM, although this isotype also recognizes proteins.

During a secondary response, Abs switch from IgM to any of the other Ig isotypes, depending on the biochemical nature of the antigen and the entrance pathway to the organism. For example, IgG is the most abundant isotype in the bloodstream, and in order for the antigens to be found by these Abs, they must first transpose barriers like the respiratory mucosa or the digestive tract. These Abs are high-affinity monomers and increase their affinity due to a hypermutation mechanism that occurs at the DNA level of plasmocytes – the activated B lymphocytes. Moreover, the isotype switch also leads to the establishment of a subset of B cells called memory cells, which will be responsible for Ab production whenever the antigen enters the organism again. In that case, IgG production will be faster and more intense; IgG has a considerable ability to activate the complement system and to bind Fc receptors of phagocytes, thus helping the immune system to remove the antigen from circulation.

The production of IgA is associated with mucosal tissues and salivary fluid. This Ig circulates as a dimer and is particularly produced to be secreted in the extracellular space mucosal tissues. Tissue locations where IgA can be found are mostly devoid of phagocytes and complement proteins; thus, IgA functions are not directly related to antigen depuration but to antigen neutralization.

The IgE isotype is mainly found associated with mast cells via their Fc surface receptor and, to a much lesser extent, free in the bloodstream. Antigen binding to associated IgE leads to mast cell degranulation, releasing biochemical mediators of inflammation and chemotaxis. Thus, this isotype is associated with allergy because the immune response to common allergens leads to “silent” progressive sensitization of mast cells with specific IgE molecules, which in turn might produce a massive

degranulation when the antigen enters the organism again. Degranulation of mast cells leads to common clinical manifestations of allergy, like respiratory compromise, generalized inflammation, edema, etc.

In this context, determining the isotype of specific antibodies gives information concerning the entrance pathway of the antigen and also about the number of times the antigen has entered the organism (single recent contact, repeated past contact). Furthermore, the correlation between the antibody isotype and clinical features developed by individuals might make it possible to understand the ability of immunoglobulins to protect the organism from any particular antigen, either virus, bacteria, or toxic compounds.

Antibodies Against Microcystins

The use of specific antibodies as biomarkers for waterborne infectious diseases has been documented elsewhere, like the paper by Exum et al. (2016). In that work, the authors point out the utility of detecting pathogen-specific antibodies during the identification of the causal agent of gastrointestinal disease caused by norovirus, **shiga toxin-producing** *E. coli*, *Cryptosporidium* spp., rotavirus, *Shigella* spp., *Giardia* spp., *Vibrio cholerae*, *Campylobacter* spp., hepatitis A, and hepatitis E.

The presence of specific antibodies against microcystins in individuals under chronic exposure to contaminated waters has not yet been conveniently studied. The lack of evidence concerning this issue is so marked that there is still no information on whether microcystins in the bloodstream – which are small molecules and probably behave as haptens – bind any carrier protein to become immunogenic. This question is still unanswered, although the molecular structure and some laboratory assays (Meissner et al. 2013) suggest that lateral groups of amino acids might be reactive and produce a covalent bond to tissues or plasma proteins, like albumin. The production of hyperimmune sera specific for MC-LR in laboratory animals suggests that conjugation with carrier proteins is needed for immunogenicity (Baier et al. 2000; Metcalf et al. 2000; Mhadhbi et al. 2006; Young et al. 2006) and that the antibodies obtained in this manner might provide protection against toxins (Nagata et al. 1995). Although these are experimental conditions, data suggests that conjugation to carrier proteins might occur spontaneously at the physiological pH, giving rise to a specific immune response.

In terms of the utility of specific antibodies as exposure biomarkers for the exposure to microcystins, to our knowledge, there is only a single published paper so far, which belongs to our research group. In that paper, a group of settlers near a lake with recurrent algal blooms were first studied using a questionnaire in order to evaluate the degree of exposure and the exposure routes, and then the alteration of biochemical parameters and the presence of specific antibodies were studied in order to provide experimental evidence of that exposure. In that paper, we demonstrated that although the alteration of the hepatic enzymes was detected in only 25% of the exposed individuals, the presence of specific antibodies against MC-LR – either IgG or IgE – could be positively used to distinguish the exposed population from a

control group (Ruibal-Conti et al. 2019). Regarding antibody values, the exposed/non-exposed ratio was 7:1 for IgE and 3:1 for IgG, thus indicating that these molecules are good biomarker candidates for the identification of MC-LR exposure.

Applications to Prognosis

In this chapter, we have reviewed the molecular structure of microcystins and the biological mechanism of their toxicity. Furthermore, we have reviewed their distribution, target organs, and their effects in terms of the alteration of biochemical parameters, analyzing literature presenting in vivo and in vitro-based experimental evidence (see Fig. 3).

Considering the need for the identification of suitable biomarkers for assessing chronic exposure to microcystins, we here discussed the use of specific antibodies to microcystins and presented the available evidence. In particular, we reviewed the only experimental paper published on this matter, published by our research group (Ruibal-Conti et al. 2019), in which the detection of specific IgG and IgE against MC-LR proved to have a stronger correlation with the exposure status of the population under study than classic biochemical parameters like hepatic enzymes.

The use of specific Abs against microcystins as exposure biomarkers may have several advantages, among which are the fact that antibody detection is relatively simple and that it could be used to distinguish acute from chronic exposure (e.g.,

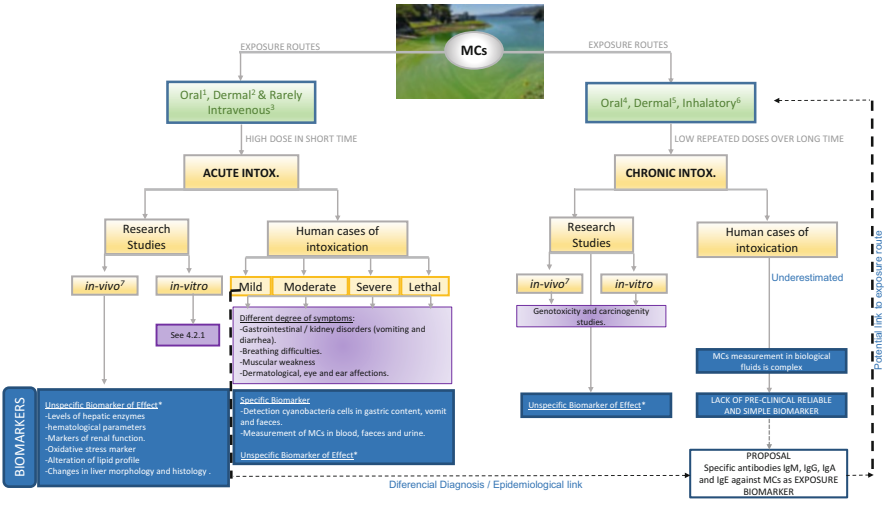

Fig. 3 General scheme for exposure routes to MCs, symptoms of acute or chronic intoxication, and potential biomarkers. 1: Generally accidental ingestion. 2: Generally by recreational or work-related skin contact. 3: Caruaru episode. 4: Generally through contaminated food, dietary supplements, or drinking water. 5: Generally by recreational, occupational, or domestic skin contact. 6: Generally inhalation of aerosols during prolonged or recurrent nautical activities or extended residence in the area. 7: Generally experiments using high doses and intraperitoneal addition of MCs

IgM in acute cases, IgG in chronic cases) if a correlation is confirmed between the isotype and exposure time. Furthermore, Abs might constitute adequate tools for the implementation of population studies and epidemiological surveillance programs.

The main obstacles related to the use of specific antibodies as biomarkers of exposure to microcystins are of a theoretical and technical nature. On one hand, there is still a considerable lack of information concerning how the immune system reacts to toxins and to what extent the exposure route shapes that response. Although the standard IgM/IgG scheme to discriminate acute from chronic conditions works for many infectious diseases, an algorithm including IgA, an isotype usually predominant in mucosal tissues, like the intestine or oral cavity, or including IgE, an isotype that is associated to allergic responses, should not be discarded. Concerning technical obstacles, it should be stated that culturing toxin-producing cyanobacteria in laboratory conditions is a delicate procedure with many difficulties and that toxin purification is a key process when designing assays for antibody detection, because any microbial contaminant is likely to produce interference in the detection system, leading to cross-reactions that impair further analyses.

Taken together, all this evidence suggests that Abs are suitable candidates for constituting exposure biomarkers of exposure to microcystins – and potentially other cyanotoxins – although further research is still needed to assess, for example, whether their titration could provide more information, and the duration of these molecules in the bloodstream or biological fluids. Moreover, there is still much research to be done regarding their protective capacity to prevent the development of clinical symptoms of intoxication.

Mini-dictionary of Terms

- **ADDA**: Unusual non-protein β-amino acid that is part of the chemical structure of microcystins and is involved in the toxicity mechanism. It is found in all microcystin congeners and also in nodularins.
- **Antigen**: A structure "foreign" to the organism that can be detected by the immune system.
- **Cyanobacteria**: Ancient unicellular microorganisms related to gram-negative bacteria but with green and blue pigments that make them capable of photosynthesis. They represent the earliest known form of life on the earth.
- **Cyanobacterial bloom**: Massive development of cyanobacteria that turns the color of water intense green.
- **Paratope**: Part of an antibody that recognizes and binds to an antigen.
- **Isotype**: Subclass of immunoglobulins.
- **Antibody**: Proteic immunoglobulin, part of the adaptive immune response.
- **Glycosylation**: Specific cell-associated pattern of surface carbohydrates.
- **Hypermutation mechanism**: A somatic mechanism in which some genes vary during cell proliferation.
- **Fc**: Constant fragment "c" of immunoglobulins.
- **Chemotaxis**: A process by which immune cells move toward their site of action.

- **Massive degranulation**: When mast cells release their secretory vesicle content.
- **Leukopenia**: A condition in which there is a lower-than-normal number of leukocytes (white blood cells) in the blood.
- **Hapten**: A "foreign" particle that cannot start an immune response de novo.
- **Thrombocytopenia**: A low platelet count.

Key Facts of Antibodies as Biomarkers: Effect of Microcystin Exposure

Key Facts of Cyanobacterial Blooms

- Cyanobacterial blooms are not always toxic. However, for risk management strategies, any cyanobacteria bloom is considered potentially toxic until toxicity is tested.
- Microcystins are secondary metabolites not excreted by cyanobacterial cells; they are released into the surrounding waters when cyanobacteria cells disintegrate due to natural or artificial adverse conditions.

Key Facts of Microcystins

- Microcystins are stable, thermoresistant, but photolabile molecules with a typical environmental half-life of 10 weeks.
- Microcystin can bioaccumulate in fish muscle and viscera.
- Microcystin-LR is classified IARC 2B, which means "possibly carcinogenic to humans," by the International Agency for Research on Cancer.

Key Facts of Human Intoxications with Microcystins

- The most relevant case of microcystin intoxication occurred in 1996 in a hemodialysis unit in Caruaru, Brazil, where 60 patients died.
- There are currently no specific treatments, antidotes, or vaccines.
- Treatment is based on decontamination if exposure is recent, supportive measures, maintenance of a patient airway, and symptomatic treatment.

Summary Points

- Toxic cyanobacteria growth in water bodies is a worldwide problem, and the concern it generates is genuine and growing.
- Microcystins are small cyclic heptapeptides produced by cyanobacteria and characterized by an ADDA group that is partly responsible for the toxicity of the molecule and might be involved in its immunogenicity.
- At the cellular level, MCs inhibit serine/threonine protein phosphatases 1 and 2A affecting many metabolic pathways, in particular multiple control mechanisms of the cell cycle.

- Microcystins are mainly hepatotoxic but may affect organs as diverse as the kidneys, nervous system, respiratory system, and reproductive organs.
- Health effects of MC-LR have been proven in cell lines and animal models, and worrying statistical associations have also been reported in epidemiological studies.
- The clinical effects of microcystin exposure depend on the route and the level of exposure, ranging from mild, severe, to lethal.
- Acute exposure to high concentrations of these toxins causes liver damage, while subchronic or chronic exposure may promote liver or colon tumor formation.
- Acute intoxication with MCs is difficult to diagnose because its symptoms and effect biomarkers are nonspecific and easily confused with those of other diseases.
- Chronic or subchronic intoxication with MCs is difficult to identify as there is no specific preclinical biomarker.
- Proposed effect biomarkers are unspecific and include hepatic enzymes, hematological parameters, oxidative stress markers, and markers of renal function.
- Isolation of the toxin in blood and tissue is complex and requires the use of sophisticated equipment.
- The use of specific antibodies as biomarkers for waterborne infectious diseases is well known but has not yet been conveniently studied in the field of toxic cyanobacterial blooms.
- Theoretical evidence indicates that antibodies against MC would be a useful, simple, and specific method/biomarker to measure or follow up exposure to microcystins, although the only existing experimental evidence is from work done by our group.
- Antibodies include different isotypes that could be indicative of the type of exposure route.
- In the future, it will be necessary to investigate the link between the different immunoglobulin isotypes and the titration and persistence of antibodies over time in order to reliably establish the potential of specific antibodies as biomarkers of exposure to cyanotoxins.

Cross-References

► Biomarkers for Assessing Mycotoxin Exposure and Health Effects
► Biomarkers of Liver Injury due to Toxic Agents: Progress, Current Applications, and Emerging Directions

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Gamma-H2AX Expression as a Biomarker of Carcinogenesis: Applications to Toxicology

6

Shugo Suzuki and Hideki Wanibuchi

Contents

Abstract

Cancer develops due to the accumulation of genetic and epigenetic changes caused by various factors. An important source of genetic changes is DNA double-strand breaks (DBSs). Radiation, oxidative stress, and carcinogens in the environment cause DNA DSBs. Cells respond to DSBs by phosphorylating histone H2AX to produce γH2AX. In *in vitro* assays, analysis of γH2AX formation enables detection of genotoxic carcinogens with a high accuracy. In *in vivo* assays, both genotoxic and non-genotoxic carcinogens can be detected. γH2AX is

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highly expressed in a variety of human precancerous and cancerous lesions. Here we explore the use of γH2AX formation as a biomarker of human tumorigenesis and as a prognostic marker for several cancers.

Keywords

γH2AX · DNA double-strand break · Radiation · Genotoxic · Non-genotoxic · Carcinogen · Cancer · Carcinogenesis · Oxidative stress · DNA repair

Abbreviations

53BP1	p53-binding protein 1
ATM	Ataxia-telangiectasia-mutated
ATR	ATM and Rad3-related
ATRIP	ATR-interacting protein
BRCA1	Breast cancer associated 1
DNA-PK	DNA-dependent protein kinase
DSBs	DNA double-strand breaks
ECVAM	European Centre for the Validation of Alternative Methods
ELISA	Enzyme-linked immunosorbent assay
HR	Homologous recombination
IARC	International Agency for Research on Cancer
LC-MS/MS	Liquid chromatography-tandem mass spectrometric
MDC1	Mediator of DNA damage checkpoint protein 1
MRE11	Meiotic recombination 11
MRN	MRE11-RAD50-NBS1
NBS1	Nijmegen breakage syndrome 1
NHEJ	Non-homologous end joining
RNF168	Ring finger protein 168
RNF8	Ring finger protein 8
ROS	Reactive oxygen species
RPA	Replication protein A
UV	Ultraviolet

Introduction

Cancer is a leading cause of morbidity and mortality worldwide. In 2017, there were 24.5 million cancer cases worldwide and 9.6 million cancer deaths. The morbidity and mortality from neoplasms are on the rise (Global Burden of Disease Cancer et al. 2019). Cancer develops due to the accumulation of genetic and epigenetic changes caused by a variety of factors. Various carcinogens are present in the environment, and people are exposed to them on a daily basis. In the field of toxicology and carcinogenesis, one of the goals is to detect carcinogenic substances in the environment and to elucidate their mechanisms of action. Therefore, it is important to identify biomarkers to detect carcinogens and factors that play an important role in carcinogenesis.

In carcinogenesis, DNA double-strand breaks (DSBs) are one of the most critical events affecting DNA. DSBs can occur when a cell or an organism is exposed to ionizing radiation or certain chemical or environmental stresses. DSBs can initiate genomic instability, ultimately leading to cancer (Bonner et al. 2008). When DSBs occur, the carboxy-terminal serine 139 of histone H2AX is phosphorylated (Rogakou et al. 1998): H2AX phosphorylated on serine 139 is known as γH2AX. Thus, increased expression of γH2AX signifies a state of genomic instability, which may contribute to the carcinogenic process. Thus, the formation of γH2AX may be a biomarker for carcinogenesis.

In this chapter, we summarize the role of histone H2AX and the function of its phosphorylated derivative γH2AX, and we validate the use of γH2AX as a biomarker of the oncogenic process.

Histone H2AX

Histones are proteins which package DNA into nucleosomes and maintain the shape and structure of a nucleosome. Histones are separated to linker histones and core histones. The linker histone H1 interacts with the DNA between nucleosomes (known as linker DNA) and functions in compacting chromatin into higher-order structures. The core histones consist H2A, H2B, H3, and H4. Two molecules of each of the four core histone proteins form an octamer of core histone proteins around which 145–147 base pairs of DNA are wrapped (Luger et al. 1997). This unit is called a nucleosome, which is the smallest subunit of genomic DNA in eukaryotic cells.

Histone H2AX is one of the variants of histone H2A (Redon et al. 2002). There are numerous H2A isoforms: 3 clusters of 17 H2A genes are mapped on chromosome 6 and chromosome 1 in humans. H2AX constitutes approximately 2.5–25% of the total H2A in mammalian cells; its levels are cell line- and tissue-dependent (Redon et al. 2002). Interestingly, H2AX has a unique function. The main function of the other H2A variants is associated with regulation of transcription, but H2AX is associated with DNA repair (Shah et al. 2020), and it is distributed throughout mammalian chromatin. Like other members of the H2A family, H2AX can be phosphorylated on Ser 1, acetylated on Lys 5, and ubiquitinated on Lys 119. But H2AX also has a unique extended C-terminal tail which contains a serine (Ser-139) embedded in an invariant SQEY motif that is a target for phosphorylation by the phosphatidylinositol 3-kinase-related DNA-dependent protein kinase (DNA-PK), the ataxia-telangiectasia-mutated (ATM) protein kinase, and the ATM and Rad3-related (ATR) protein kinase (Bonner et al. 2008).

Phosphorylation of H2AX (γH2AX)

The phosphorylation of the Ser139 in the SQEY motif of H2AX immediately after the introduction of DNA damage in human cells was first reported in 1998 (Rogakou et al. 1998). The Ser139-phosphorylated form of H2AX was named γH2AX because it was observed in cells exposed to ionizing radiation such as γ-rays.

When a DNA DSB occurs, specific proteins bind to the DNA ends and recruit one of the three PI3-kinases, ATR, DNA-PK, or ATM, to the DSB site (Blackford and Jackson 2017). Which proteins bind to the free DNA end of a DSB and which PI3-kinase is recruited depend on the cause and timing of the DNA damage (Bonner et al. 2008). When a DBS occurs during DNA replication, the DNA ends are processed to generate regions of single-stranded DNA. The single-stranded DNA is bound by replication protein A (RPA). ATR-interacting protein (ATRIP) then binds to the RPA coating the single-stranded DNA, and ATR binds to ATRIP (Blackford and Jackson 2017). DNA ends that are not processed to generate single-stranded DNA are bound by the Ku70/80 and the meiotic recombination 11 (MRE11)-RAD50-Nijmegen breakage syndrome 1 (NBS1) (MRN) complexes (Blackford and Jackson 2017). The Ku70/80 complex is highly abundant in eukaryotic cells, and when it encounters a DNA DSB, it binds the free DNA ends and recruits DNA-PK to the site of DNA damage (Blackford and Jackson 2017). Ku70/80 and DNA-PK form a complex containing a DNA binding tunnel that accommodates approximately 30 bp of DNA, stabilizing the DNA end (Yin et al. 2017). Similarly to Ku70/80, the MRN complex is dispersed throughout the nucleus, but unlike Ku70/80, which scans for free DNA ends via three-dimensional diffusion, MRN scans along the DNA, a process known as one-dimensional facilitated diffusion (Myler et al. 2017). When the MRN complex encounters a free DNA end, it binds the DNA and recruits ATM to the site of DNA damage (Blackford and Jackson 2017). Depending on other factors, including cell cycle stage, if MRN encounters DNA ends already bound by Ku70/80, it can remain bound to the DNA and recruit ATM and enhance DNA repair by non-homologous end joining (NHEJ), or it can remove the Ku70/80-DNA-PK complex and promote resection of the DNA and binding of RPA and ART-ATRIP to the resultant single-stranded DNA and promote DNA repair by homologous recombination (HR) (Oh and Symington 2018; Reginato and Cejka 2020; Shibata et al. 2018; Syed and Tainer 2018). After recruitment to sites of DNA damage, ATR, DNA-PK, and ATM are activated and phosphorylate a number of substrates including histone H2A. Phosphorylation of H2A at Ser-139 generates γH2AX. γH2AX generation is one of the earliest events detected in cells following exposure to DNA-damaging agents; it appears within minutes and reaches maximum levels after 30 min (Rogakou et al. 1999).

Thus, DNA DSBs can initiate genomic instability that ultimately leads to cancer, and DNA DSBs also trigger activation of protein kinases that phosphorylate H2AX at Ser-139. Events that cause DSBs, triggering generation of γH2AX, include direct attack on DNA by reactive oxygen species (ROS), metabolic processes, DNA repair defects, and telomere erosion, and these events are involved in carcinogenesis (Bonner et al. 2008). Therefore, generation of γH2AX could be a biomarker of an event having the potential to promote carcinogenesis.

Functions of γH2AX

γH2AX has been reported to play an essential role in DNA DSB repair and genomic stability and is classified as a tumor suppressor. Upon phosphorylation of H2A, mediator of DNA damage checkpoint protein 1 (MDC1) binds to the newly generated γH2AX. Subsequently, additional MRN-ATM complexes bind to the MDC1 tethered to the γH2AX. Recruitment of additional MRN-ATM to the DNA DSB permits phosphorylation of additional H2AX proteins by ATM, generating a focus of γH2AX proteins on the damaged DNA. Recruitment and activation of ring finger protein 8 (RNF8) and ring finger protein 168 (RNF168) activate a phosphorylation-ubiquitylation signalling cascade that results in recruitment of additional DNA repair and/or checkpoint proteins to the γH2AX focus; γH2AX is also involved in the recruitment of chromatin remodeling complexes and chromatid cohesins (Blackford and Jackson 2017; Bonner et al. 2008; Ivashkevich et al. 2012). Accordingly, depending on a number of factors, including cell cycle stage and interactions between the proteins recruited to the damaged DNA, proteins that promote NHEJ repair or proteins that promote repair of DSBs by HR or proteins that promote other pathways will be recruited to γH2AX foci. The various repair and signalling pathways involving γH2AX are presented in Fig. 1 (Ivashkevich et al. 2012). Thus, γH2AX formation is involved in gene repair and stability and acts as a tumor suppressor. This is evidenced *in vivo* by the finding of chromosomal instability, repair defects, and impaired mobilization of NBS1, p53-binding protein 1 (53BP1), and breast cancer associated 1 (BRCA1) to irradiation-induced foci in H2AX knockout mice (Celeste et al. 2002).

Several other functions of γH2AX have been reported: these include sex chromosome inactivation in male germ cells, X chromosome inactivation in female somatic cells, chromatin regulation during mitosis, embryonic and neural stem cell development, asymmetric sister chromosome segregation in stem cells, and maintenance of cellular senescence. The modification of H2AX in contexts other than the DNA damage response may contribute towards creating a specific chromatin structural frame allowing various different functions to be carried out (Turinetto and Giachino 2015).

Methods of γH2AX Detection

The main techniques for measuring the amount of γH2AX use antibodies against phosphorylated serine 139. This type of detection is used for immunostaining, flow cytometry, Western blotting, and enzyme-linked immunosorbent assay (ELISA) (Palla et al. 2017; Ji et al. 2017). Detection methods can be divided into counting foci and other γH2AX-containing structures in cell and tissue images and measuring overall γH2AX protein levels (Palla et al. 2017; Bonner et al. 2008).

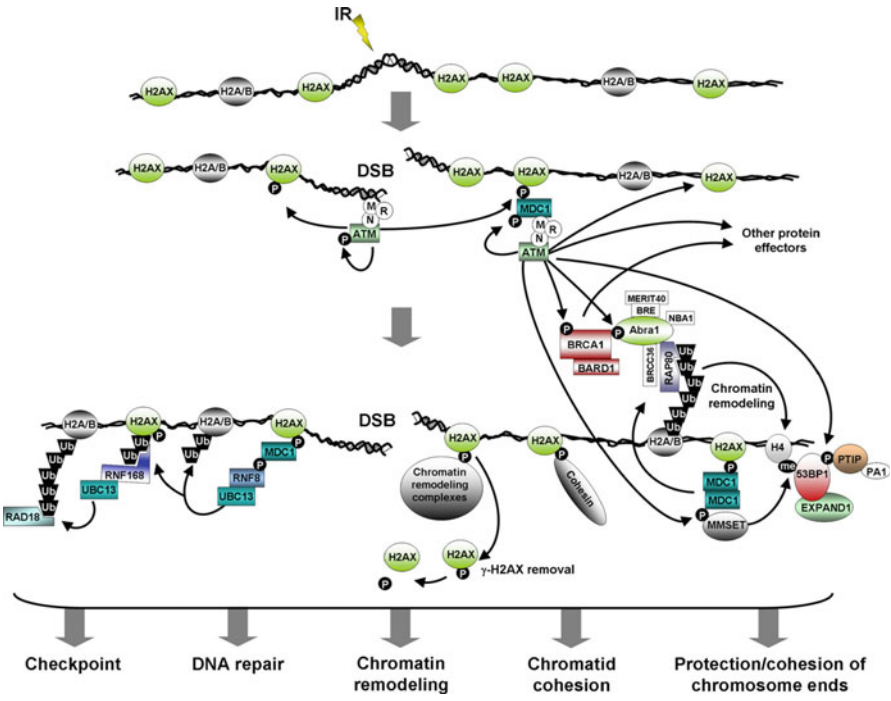

Fig. 1 γH2AX formation and its role in the irradiation-induced DNA repair foci. Foci assembly is a hierarchical process which starts with DSB recognition by the MRN complex, recruitment of the ATM kinase, and its autophosphorylation at the DSB site. The resulting ATM-mediated γH2AX allows the recruitment of MDC1. MDC1 binding to γH2AX, in turn, allows the recruitment of other proteins including the MRN complex and ATM. Additional recruitment of ATM will then permit phosphorylation of additional H2AX and other DNA repair proteins at the DSB (RNF8, BRCA1, 53BP1, etc.). MDC1 also recruits RNF8, an ubiquitin ligase which initiates histone H2 (H2AX, H2A, H2B) poly-ubiquitylation at DSB sites. These histone modifications stimulate a second wave of protein accumulation, including proteins and/or protein complexes such as the BRCA1 A complex, 53BP1, RAD18, PTIP, EXPAND1, etc. γH2AX is also involved in the recruitment of chromatin remodeling complexes and chromatid cohesins. Foci formation is thought to stimulate DNA repair and checkpoint activation, to allow chromatin remodeling and sister chromatid cohesion, and to facilitate cohesion of broken chromosome ends. P, phosphate; Ub, ubiquitin; me, methyl; M, MRE11; N, NBS1; R, RAD50. To simplify, single histones are shown. (Reprinted from Cancer Letters, 327 (1-2), Ivashkevich A, Redon CE, Nakamura AJ, Martin RF, and Martin OA, Use of the gamma-H2AX assay to monitor DNA damage and repair in translational cancer research, 123-133, Copyright (2012), with permission from Elsevier.)

Immunocytochemical detection of γH2AX foci is highly sensitive and can distinguish between pan-nuclear staining and foci formation; flow cytometry measures the number of cells with given levels of γH2AX; and Western blotting and ELISA measure total γH2AX in a sample. In addition, a technique has recently been reported that can quantify γH2AX using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Qu et al. 2020). LC-MS/MS provides a sensitive and specific analysis of histone peptides to determine the relative levels of core

histones and their posttranslational modifications. It is a powerful tool for quantifying the relative levels of histone-modifying factors. The development of these detection methods has increased the usefulness of γH2AX as a biomarker.

The Relationship Between γH2AX Formation and Carcinogens

Cancer develops due to the accumulation of genetic and epigenetic changes caused by various factors. It is also generally believed that cancer development is a multistep process, and this has been confirmed by studies on colorectal cancer (Fearon and Vogelstein 1990). This process involves multiple molecular and cellular events that lead to the transformation of normal cells into malignant neoplastic cells (Klaunig et al. 2010, 2011). Carcinogenesis induced by chemical toxins can be divided into three steps, initiation, promotion, and progression (Fig. 2) (Klaunig et al. 2011). Detecting the substances involved in each step of this carcinogenesis process is an important field of study.

Based on the results of epidemiological and animal studies, carcinogens are classified as Group 1 (carcinogenic to humans), Group 2A (probably carcinogenic to humans), Group 2B (possibly carcinogenic to humans), or Group 3 (not classifiable as to its carcinogenicity to humans). Carcinogens with sufficient evidence of carcinogenicity in humans do not need any further evidence of their carcinogenicity. However, it can be argued that carcinogens with limited or inadequate evidence of cancer in humans are the more crucial carcinogens as it is unknown how to regulate these agents. A critical factor when classifying these agents is determining if the mechanism by which they induce carcinogenesis operates in humans. Carcinogens

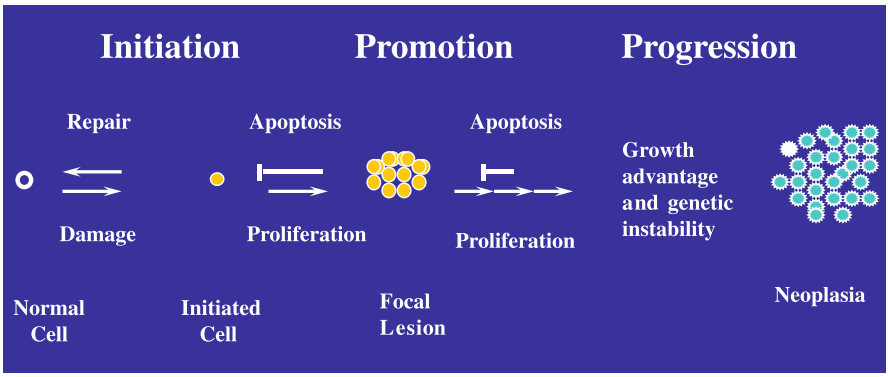

Fig. 2 Multistage carcinogenesis. Cancer induction is a multistep process in which multiple molecular and cellular events lead to the transformation of normal cells into malignant neoplastic cells through the accumulation of genetic and epigenetic changes. Among them, at least three steps (carcinogenesis, promotion, and progression) have been defined for the chemical carcinogenesis process. (Reprinted from Toxicology and Applied Pharmacology, 254, Klaunig JE, Wang Z, Pu X, and Zhou S, Oxidative stress and oxidative damage in chemical carcinogenesis, 86-99, Copyright (2011), with permission from Elsevier)

that cause DNA damage, thereby inducing the formation of γH2AX, in experimental animals have the potential to cause DNA damage and promote carcinogenesis in humans. Below we discuss some of the major carcinogens that have been reported to induce the formation of γH2AX.

Radiation

Ionizing radiation and ultraviolet (UV) radiation are well-known inducers of somatic mutations and cancers. These types of radiation can induce a broad spectrum of DNA lesions including damage to nucleotide bases, cross-linking, and DNA single-strand break and DSBs (Little 2000). Notably, γH2AX was first detected in cell cultures and mice exposed to cesium-137, a beta and gamma ray emitter (Rogakou et al. 1998). The DSBs induced by radiation correlated with γH2AX detection in the nucleus (Rogakou et al. 1999). Subsequently, γH2AX expression was reported to be a biomarker of DNA damage after exposure of patients and animals to radiation (Fernandez-Palomo et al. 2015; Alipoor et al. 2018; Mah et al. 2010). Therefore, γH2AX formation is a biomarker that detects the toxicological effects of radiation.

Oxidative Stress

ROS are induced through a variety of endogenous and exogenous sources. Endogenous sources include mitochondria, peroxisomes, and inflammatory cell activation. Exogenous sources include environmental agents including radiation, genotoxic chemicals (N-nitroso compounds, heterocyclic amines, etc.), non-genotoxic chemicals (2-butoxyethanol, chlorinated compounds, peroxisome proliferators, etc.), and tobacco smoke (Klaunig et al. 2011).

Overwhelming the antioxidant and DNA repair mechanisms in the cell by excessive ROS can result in oxidative stress and oxidative damage to the cell. The resulting oxidative stress can damage critical cellular macromolecules and/or modulate gene expression pathways. DNA damage induced by ROS includes DNA single-strand breaks, base modifications, deoxyribose modifications, and DNA cross-linking (Mah et al. 2010; Klaunig et al. 2011) and DNA double-strand breaks, which enhances γH2AX formation. Therefore, since excessive ROS formation can cause DNA DSBs leading to γH2AX formation and ultimately to carcinogenesis, γH2AX formation can be a useful biomarker for the detection of this form of oxidative stress-mediated carcinogenicity (Mah et al. 2010; Klaunig et al. 2011).

Chemicals

There are many chemicals that have been identified as carcinogens in humans and animal models: many of these chemicals are summarized in monographs by the IARC (https://monographs.iarc.who.int/). Weisburger et al. published a paper in

which they divided chemical carcinogens into two classes, genotoxic and non-genotoxic, based on the reactivity of the chemical with DNA (Weisburger and Williams 1981). The chemical carcinogens that react directly with DNA or react with DNA following metabolic activation are categorized as genotoxic carcinogens. DNA adduct levels induced by genotoxic carcinogens reflect tissue-specific rates of damage processing that include DNA adduct formation and removal (DNA repair), DNA adduct instability, tissue turnover, and other events. DNA damage caused by genotoxic carcinogens that results in DSBs will lead to γH2AX formation. Therefore, γH2AX formation is a useful biomarker for these types of genotoxic carcinogens.

Numerous chemicals can cause DNA damage indirectly. For example, as noted above, a variety of chemicals cause the formation of ROS, and this can result in DNA DSBs. Therefore, whether these chemicals are classified as non-genotoxic or genotoxic, γH2AX is a biomarker of these types of chemical carcinogens.

γH2AX Formation as Genotoxic Biomarker in *In Vitro* Studies

In vitro experiments have been conducted using γH2AX formation as a biomarker for carcinogen detection (Nikolova et al. 2014; Tsamou et al. 2012). An initial study had relatively low sensitivity, detecting 13 of 24 genotoxic carcinogens tested (Tsamou et al. 2012); however, a later study detected 14 of 14 genotoxic carcinogens tested (Nikolova et al. 2014). Both of these *in vitro* studies showed that the cytotoxicity of the genotoxic carcinogens was correlated with γH2AX formation while non-genotoxic chemicals did not induce γH2AX formation. Recently, in a study using LC-MS/MS to quantify γH2AX formation, 69 of the chemicals recommended by the European Centre for the Validation of Alternative Methods (ECVAM) that have already been shown to be genotoxic were tested. The results showed a high sensitivity (88%) and specificity (100%), detecting 22 out of 25 genotoxic substances. Additionally, the dose-effect relationship between genotoxic substances and γH2AX formation was ascertained. When the genotoxicity mechanisms were classified and analyzed separately, five DNA alkylating agents, seven DNA adduct agents, three nucleoside analogues, and five other genotoxic agents were all detectable by γH2AX assay. On the other hand, only two of the five aneugenic agents, i.e., genotoxicants that act primarily on non-DNA targets, were detectable (Qu et al. 2021).

The data obtained by the three *in vitro* experiments referenced above are summarized in Table 1. These data suggest that the development of research methods has

Table 1 Summary of γH2AX detection of genotoxic chemicals

Author	Year	Method	Sensitivity	Specificity
Tsamou et al.	2012	Flow cytometry	13/24 (54%)	31/40 (78%)
Nikolova et al.	2014	Flow cytometry	14/14 (100%)	10/10 (100%)
Qu et al.	2021	LC-MS/MS	22/25 (88%)	44/44 (100%)

increased the usefulness of γH2AX as a biomarker, and γH2AX has been shown to be useful as an *in vitro* detection method for genotoxic carcinogens.

γH2AX Formation as Biomarker of Carcinogen in *In Vivo* Studies

In vivo studies have also been performed using the γH2AX assay as a method to detect carcinogens, especially bladder carcinogens. Toyoda and collaborators showed that genotoxic bladder carcinogens significantly increased the number of γH2AX-positive urothelial cells in a 4-week rat study (Toyoda et al. 2015), indicating that γH2AX is a useful biomarker for the early detection of genotoxic bladder carcinogens in rats. Recently, it was reported that some non-genotoxic bladder carcinogens also induced γH2AX-positive urothelial cells in rats (Yamada et al. 2020, 2021; Suzuki et al. 2020). These data are summarized in Table 2. When considering the mechanisms of carcinogenesis, toxic effects such as calculi, oxidative stress, and/or inflammation by non-genotoxic carcinogens are important factors. Such toxic effects can produce γH2AX formation (Arnold et al. 1997; Fujii et al. 1987; Cohen 1998). Additionally, there was a dose-response of γH2AX generation in the urothelium of rats treated with both genotoxic and non-genotoxic bladder carcinogens (Yamada et al. 2020). These findings suggested that the toxic effect of non-genotoxic bladder carcinogens may cause DNA DSBs, suggesting that γH2AX may be a useful biomarker for bladder carcinogens regardless of genotoxicity; however, further investigation of this point is required.

Attempts to detect carcinogens by γH2AX assay have been made in other organs. Hepatic carcinogens (2-acetylaminofluorene and *N*-nitrosodiethylamine) induced γH2AX formation in hepatocytes, but carcinogens to the thyroid gland (glycidol and acrylamide) did not induce the formation of γH2AX in follicular epithelial cells (Toyoda et al. 2015). The gastric carcinogen N-methyl-N-nitrosourea induced γH2AX formation in the gastric mucosa, but carcinogens which target

Table 2 Summary of γH2AX detection of rat bladder carcinogens and noncarcinogens. In several instances these studies use data for the same chemical. Therefore, the number of chemicals in the Summary does not match the total chemicals shown in the study list

		Sensitivity			Specificity
Author	Year	GTBC	NGTBC	BC (GTBC + NGTBC)	BNC
Toyoda et al.	2015	4/4 (100%)	0/3 (0%)	4/7 (57%)	3/3 (100%)
Toyoda et al.	2019	3/3 (100%)	–	3/3 (100%)	3/3 (100%)
Yamada et al.	2020	1/1 (100%)	1/1 (100%)	2/2 (100%)	–
Suzuki et al.	2020	3/3 (100%)	6/7 (86%)	9/10 (90%)	4/5 (80%)
Yamada et al.	2021	9/11 (82%)	2/3 (67%)	11/14 (79%)	5/5 (100%)
Summary[a]		14/16 (88%)	8/10 (80%)	22/26 (85%)	12/13 (91%)

GTBC genotoxic bladder carcinogen, *NGTBC* non-genotoxic bladder carcinogen, *BC* bladder carcinogen, *BNC* bladder noncarcinogen

[a]In several instances these studies use data for the same chemical. Therefore, the number of chemicals in the Summary does not match the total chemicals shown in the study list

non-stomach tissues did not (Okabe et al. 2019). These data suggest that γH2AX may be a useful biomarker for the detection of carcinogens in specific, but not all, organs. Further investigation of carcinogen detection by γH2AX formation in different organs is needed.

γH2AX Formation as Biomarker in Clinical Studies

DNA damage and genomic stability are well-known factors associated with the progression from normal to premalignant and to malignant tissues. A high expression of γH2AX was detected in breast ductal carcinoma, hepatocellular carcinoma, endocervical and vulva/penis squamous carcinoma, gastric carcinoma, colorectal cancer, thyroid cancer, epithelial ovarian cancer, and melanoma (Palla et al. 2017; Saravi et al. 2020; Manoel-Caetano et al. 2019). γH2AX was also increased in the preneoplastic lesions of hepatocellular carcinoma and cervical squamous cell carcinoma, oral epithelial dysplasia, and colonocytes from ulcerative colitis patients, a chronic inflammatory disease that predisposes patients to colorectal cancer (Palla et al. 2017; Leung et al. 2017). These reports indicate that γH2AX formation is also enhanced during human tumor progression, suggesting that the protracted presence of γH2AX foci may indicate an abnormally high rate of DNA damage or an abnormality in one of the processes involved in DSB repair. Thus, γH2AX may be a useful biomarker for the risk of a lesion developing into a cancer and for the early detection of carcinogenic lesions.

γH2AX as a Prognostic Marker in Several Cancer Types

In human cancers, it is important to develop markers for the early detection of carcinogenic lesions and markers that can serve as prognostic indicators. There are many reports that suggest that γH2AX can be used as a prognostic marker. A high expression of γH2AX is associated with classical prognostic factors such as tumor stage and/or histological grade in endometrial carcinoma, breast cancer, hepatocellular carcinoma, and thyroid cancer (Palla et al. 2017). A high γH2AX expression was also associated with decreased overall survival in colorectal cancer, non-small cell lung carcinoma, oral squamous cell carcinoma, and hepatocellular carcinoma after liver transplantation (Palla et al. 2017). DNA damage and genomic stability are well-known factors associated with cancer progression, and as noted above, the presence of high levels of γH2AX foci may indicate an abnormally high rate of DNA damage or an abnormality in one of the processes involved in DSB repair. Therefore, it is understandable that formation of γH2AX is a useful prognostic marker in some cancers.

On the other hand, since γH2AX formation is related to DSB repair and genomic stability, decreased γH2AX formation may indicate a decrease in DNA DSB repair and an increase in the mutation rate in the affected tissue and accelerated progression of the developing cancer. The lack of γH2AX in low-grade urothelial carcinoma was

associated with a statistically significantly higher rate of recurrence than in γH2AX positive-cases. Similarly, γH2AX positivity was found to be associated with a lower rate of tumor recurrence in urothelial cancer patients, suggesting prevention of the accumulation of genomic damage (Palla et al. 2017; Cheung et al. 2009). In terms of prognostic value, a higher expression of γH2AX was associated with better overall survival in ovarian cancer (Saravi et al. 2020). In summary, γH2AX formation is useful as a prognostic marker for a variety of cancers, but because its expression can vary from organ to organ and it may indicate increased DNA repair or increased DNA damage/defective DNA repair, further studies to enable determination of the cause of abnormal levels of γH2AX in each tumor are required.

Conclusion

γH2AX, which is formed as a consequence of protein complexes that bind to DNA DSBs, is a marker of DNA DSB repair. It is therefore a biomarker of a mechanism known to be involved in carcinogenesis. *In vitro*, it was reported that γH2AX could detect genotoxic carcinogens with a high accuracy, and *in vivo*, it was shown that γH2AX could detect both genotoxic and non-genotoxic carcinogens. γH2AX is highly expressed in various human precancerous and cancerous lesions, indicating not only that it is useful as a biomarker for tumorigenesis but also that γH2AX expression can serve as a prognostic marker in some cancers. However, γH2AX can indicate the presence of high rates of DNA damage or an abnormality in DNA repair and be associated with a worse prognosis, or γH2AX can indicate the process of DNA repair and be associated with a better prognosis. Therefore, it is important to perfect methods of verifying these alternative fates of γH2AX when it is found to be present at abnormal levels.

Mini-dictionary of Terms

- **Carcinogen.** It is an agent with the capacity to cause cancer.
- **Double-stranded DNA breaks.** It is a harmful event because it can cause genomic rearrangements when both DNA strands are broken.
- **The European Centre for the Validation of Alternative Methods (ECVAM).** It is a unit of the Institute for Health and Consumer Protection of the European Commission's Joint Research Centre with a view to the development, validation, and international recognition of alternative test methods.
- **The International Agency for Research on Cancer (IARC).** It is the specialized cancer agency of the World Health Organization and promotes international collaboration in cancer research.
- **The IARC Monographs.** They present environmental factors that are carcinogenic hazards to humans based on epidemiological studies and animal experiments.

Key Facts of Genotoxicity

- Genotoxicity describes the property of agents that can cause DNA or chromosomal damage.
- It can lead to mutations and, possibly, various types of cancer.
- *In vitro* and *in vivo* assays to detect genotoxicity have been established.
- *In vitro*, there are genotoxicity assays such as the Ames test and the mouse lymphoma test.
- *In vivo*, there are genotoxicity assays such as chromosome aberration assay and micronucleus assay.

Summary Points

- DSBs, the cause of γH2AX formation, are induced by direct attack on DNA by ROS, metabolic processes, and repair defects and are thought to be involved in carcinogenesis.
- γH2AX plays an essential role in DSB repair and genomic stability and is classified as a tumor suppressor.
- Using γH2AX formation as an indicator, genotoxic carcinogens could be detected with a high accuracy in *in vitro* studies.
- γH2AX assay detects not only genotoxic bladder carcinogens but also non-genotoxic bladder carcinogens in *in vivo* studies.
- γH2AX is highly expressed in various human precancerous and cancerous lesions.
- γH2AX expression can serve as a prognostic marker in some cancers.

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The Carcinogen Glycidol and Use of *N*-(2,3-Dihydroxypropyl)-valine in Hemoglobin as a Biomarker of Exposure

7

Bernhard H. Monien and Klaus Abraham

Contents

Abstract

Fatty acid esters of glycidol (glycidyl esters, GE) are processing contaminants in vegetable oils and fats. GE release the carcinogenic glycidol in the gastrointestinal tract. The assessment of health risks associated with dietary GE uptake is hindered by the inaccuracy of exposure estimations based on consumption and food content data. Alternatively, the internal exposure can be approximated by monitoring of human biomarkers of glycidol, for example, the hemoglobin

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adduct *N*-(2,3-dihydroxypropyl)-valine (DHP-Val). The quantification of DHP-Val levels in blood samples showed that human adults are exposed principally by foodstuffs and tobacco smoke. Reverse dosimetry allowed calculating the mean oral exposure for 11 German adults (0.94 μg/kg body weight) and 50 Swedish adolescents (1.4 μg/kg body weight). These values exceeded the median chronic exposure estimated from dietary surveys for the adult population (0.2 μg/kg body weight) and for adolescents (0.3 μg/kg body weight), which may be due to hitherto unknown sources of glycidol/GE. Data on DHP-Val in strict raw food eaters, who do not consume food heated to more than 42 °C, suggests that DHP-Val is also formed independently from the oral exposure to GE.

Keywords

Glycidol · Glycidyl ester · Adducts · Hemoglobin · Edman degradation · UHPLC-MS/MS · Human biomonitoring · Biomarker · Exposure · Reverse dosimetry

Abbreviations

DHP-Val	*N*-(2,3-dihydroxypropyl)-valine
DHP-Val-FTH	*N*-(2,3-dihydroxypropyl)-valine fluorescein thiohydantoin
EFSA	European Food Safety Authority
FITC	Fluorescein-5-isothiocyanate
FTH	Fluorescein thiohydantoin
GC-MS	Gas chromatography-mass spectrometry
GE	Glycidyl esters
Hb	Hemoglobin
LOD	Limit of detection
LOQ	Limit of quantification
SPE	Solid-phase extraction
UHPLC-MS/MS	Ultra-high performance liquid chromatography-tandem mass spectrometry

Introduction

Occurrence and Toxicological Relevance of Glycidol

Glycidyl esters (GE) are fatty acid esters of glycidol (2,3-epoxy-1-propanol). The compounds are heat-induced food contaminants predominantly formed from acylglycerols during industrial deodorization of vegetable oils and fats involving high temperatures (Pudel et al. 2011). The highest levels of ester-bound glycidol were observed in the food groups “fats and oils” (“palm oil/fat”: 3955 μg/kg glycidol), “margarine, normal fat” (582 μg/kg glycidol), “potato crisps” (110 μg/kg glycidol), “hot surface cooked pastries” (137 μg/kg glycidol), “cookies” (134 μg/kg glycidol), and “short crusts” (149 μg/kg glycidol) (European Food Safety

Authority 2016). The toxicological relevance regarding dietary exposure is due to the almost complete and rapid hydrolysis of GE by lipase activity in the gastrointestinal tract as observed in rats (Appel et al. 2013). The glycidol released is an electrophilic, alkylating agent which is prone to react with cellular nucleophiles. It induces gene mutations, chromosomal aberrations, sister chromatid exchange, and unscheduled DNA synthesis in vitro (reviewed by Bakhiya et al. (2011)). After intraperitoneal treatment with glycidol (150 mg/kg body weight), an increased micronuclei formation was observed in bone marrow of B3C3F1 mice. Glycidol is a multisite carcinogen inducing various tumors, for example, mesothelioma in tunica vaginalis and peritoneum, brain glioma, and mammary gland adenocarcinoma, after administration by gavage to B6C3F1 mice (25 and 50 mg/kg body weight) and Fischer 344 rats (37.7 and 75 mg/kg body weight) for 2 years (National Toxicology Program 1990). Glycidol is classified by the International Agency for Research on Cancer (IARC) as *probably carcinogenic to humans* (group 2A) (International Agency for Research on Cancer 2000) and as a category 2 carcinogen by the Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area (MAK) in Germany (MAK Commission 2021).

Human Exposure and Aspects of Hazard Characterization

The mean daily dietary exposure to glycidol was estimated to be about 0.2 μg/kg body weight for European *adults* and 0.5 μg/kg body weight for high consumers. In comparison, *children* (1–10 years old) have an approximately three-fold higher exposure. A mean daily exposure of 1.9 μg/kg body weight (95th percentile: 4.9 μg/kg body weight) was determined for nonbreastfed *infants* exclusively fed with infant milk formula (European Food Safety Authority 2016).

Due to the almost complete hydrolysis of GE in the gastrointestinal tract, it can be assumed that the health risk potentials associated with the intake of GE or of equivalent amounts of free glycidol are similar (Abraham et al. 2013). The genotoxic and carcinogenic properties of the epoxide do not permit deriving a safe threshold value. In these cases, the risk assessment is confined to the application of the margin of exposure (MoE) concept devised by the European Food Safety Authority (EFSA). An MoE is the ratio of the lowest dose inducing a significant increase of tumor incidence in animals and exposure estimates in humans (European Food Safety Authority 2005). Using the T25, the glycidol dose that results in a 25% increase of the lifetime tumor incidence above background, determined for the induction of peritoneal mesothelioma in male rats (10.2 mg/kg body weight), the MoE indicates an only low health concern if higher than 25,000. According to the EFSA exposure estimations, this is the case for median and high exposure scenarios of *infants*, *toddlers*, and *other children (3–10 years)* and high dietary exposure of *adolescents* and *adults* in Europe. In the particular scenario of *infants* receiving formula as sole diet, the MoE was 5400 and 2100, taking into account the medium and high glycidol contents in infant formulae, respectively, raising health concerns about the substitute diet (European Food Safety Authority 2016). Responding to these results, the

European Commission has established maximum values for bound glycidol in vegetable oils and fats, as well as in infant and follow-on formula in 2018 (Commission Regulation (EU) 2018/290).

Exposure Assessment of Glycidol

The value of the MoE calculation depends on the quality of the human exposure estimation, which is beset with various sources of uncertainties: (1) The number of analytical results (samples) reported to the EFSA were limited, (2) analytical data for some food groups were lacking altogether, and (3) there may be unknown levels of free glycidol in food. The data on glycidol contents collected by the EFSA are based on the analyses of GE; suitable methods for the quantification of free glycidol were not available (European Food Safety Authority 2016). In addition, the actual exposure may be altered greatly by formation of glycidol and GE during domestic food processing and by additional uptake via alternative routes (e.g., passive smoking). The problems related to the inaccuracy of external exposure assessment can be circumvented using a human biomarker, which allows monitoring the individual internal exposure to specific substances regardless of the intake route. The current chapter summarizes results from recent studies on the principal biomarker of glycidol exposure, the hemoglobin (Hb) adduct *N*-(2,3-dihydroxy-propyl)-valine (DHP-Val), and the significance to the exposure estimation in human studies.

Biomarkers of Glycidol Exposure: Methodological Considerations

Biomarkers of Short- and Medium-Term Exposure

As an alternative to the traditional exposure assessment by dietary surveys, the individual internal exposure to reactive substances can be estimated by specific biomarkers in different matrices. For example, mercapturic acids of electrophilic metabolites formed after the detoxifying glutathione conjugation are detectable in the urine. The amounts typically reflect the exposure to the parent compound over a short period of 1–2 days (Mathias and B'Hymer 2016). As a potential biomarker of exposure for glycidol and GE, the urinary metabolite 2,3-dihydroxypropyl mercapturic acid (DHPMA) was discussed, because it was detected in rat urine after administration of high doses of glycidol and GE (Barocelli et al. 2011). However, the urinary excretion in human (exposed to much lower doses) was found at high and constant levels, which are rather independent from the intake of glycidol and GE in normal dietary quantities, led to the conclusion that DHPMA is not a suitable biomarker of short-term exposure (Abraham et al. 2021; Monien and Abraham 2022).

The adduct levels in the blood proteins serum albumin (SA) and Hb reflect the systemic exposure to reactive substances within the last 3 weeks (SA) or the last

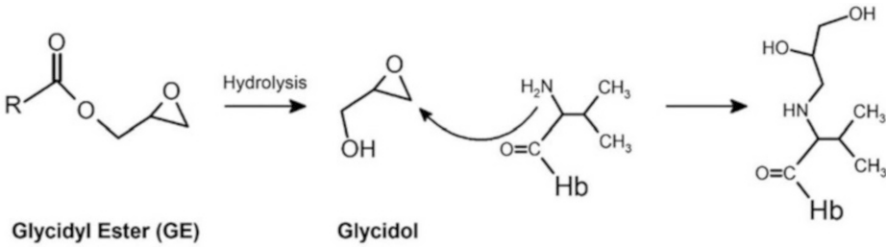

Fig. 1 Hydrolysis of glycidyl esters (GE) and reaction of glycidol with hemoglobin (Hb). Glycidol is formed by lipase-catalyzed hydrolysis of glycidyl esters (GE); *R* represents fatty acid side chains. The terminal epoxide carbon of glycidol reacts via an addition reaction with the N-terminal valine of one of the Hb peptide chains

4 months (Hb) (Sabbioni and Day 2022). The preferred choice for protein adduct analysis in the last three decades was Hb, which is abundant (~150 mg/ml blood), readily accessible and allows cumulative (medium-term) exposure monitoring due to the lifetime of aprox. 110 days (Törnqvist et al. 2002). Glycidol reacts with the N-terminal valine in the peptide chains α and β of Hb to yield the adduct DHP-Val (Fig. 1). The protein fold of Hb supports the reaction. The amino group of free valine ($pK_a = 9.74$; $pK_a = -\log_{10} K_a$, with K_a as the acid dissociation constant) is not very reactive because it is largely protonated in blood (pH = 7.4). In the environment of Hb, the valine residues are somewhat shielded. The hydrophobic environment leads to increased nitrogen acidities of the N-terminal valine in subunits α (pKa = 7.8) and subunit β (pKa = 6.8), which in turn explains the reactivity of the α-nitrogen in the valine residues, readily forming adducts with a variety of electrophilic compounds (Törnqvist et al. 2002).

Edman Degradation and Mass Spectrometric Quantification of DHP-Val in Hb

The conception for the application of Hb as a target (*dosimeter*) for the analysis of alkylating agents was introduced by Ehrenberg et al. (1974). The development of the modified Edman degradation by Törnqvist et al. was a major breakthrough in 1986, which allowed the reliable determination of Hb adducts on the N-terminal valine (Törnqvist et al. 1986). The sample preparation included several steps: isolation of globin from erythrocytes, cleavage of modified valine residues by pentafluorophenyl isothiocyanate (PFPITC), and derivatization of free hydroxyl groups of the resulting thiohydantoin using, for example, acetic anhydride (Landin et al. 1997). Optionally, the analytes were enriched by extraction with ether (Törnqvist et al. 1986) or by solid phase-extraction (SPE) (Fennell et al. 2005). Finally, the thiohydantoins were analyzed by gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-tandem mass spectrometry (LC-MS/MS).

In early studies on DHP-Val, Landin et al. (1997) and Honda et al. (2014) used the technique of PFPITC-cleavage from isolated globin samples with subsequent GC-MS analysis. Recently, the analysis of DHP-Val was improved greatly by implementing the FI*R*E procedure™ for a modified Edman degradation (Rydberg et al. 2009). The valine adduct is cleaved with fluorescein-5-isothiocyanate (FITC), resulting in the formation of *N*-(2,3-dihydroxypropyl)-valine fluorescein thiohydantoin (DHP-Val-FTH, Fig. 2). This analyte is quantified using an ultra-high performance liquid chromatography-tandem mass spectrometry system (UHPLC-MS/MS) and DHP-Val-d_7-FTH as reference standard (Hielscher et al. 2017). The technique surpasses the former methods of DHP-Val analysis due to its simplicity, sensitivity, and accuracy. The tedious globin isolation is obsolete, because the FITC-mediated valine cleavage is feasible using erythrocytes or even whole blood sample, and the resulting thiohydantoin DHP-Val-FTH (Fig. 2) does not require further derivatization for the analysis by UHPLC-MS/MS. The limit of detection (LOD; 0.7 pmol/g Hb) and the limit of quantification (LOQ; 1.4 pmol/g Hb) of DHP-Val analysis after cleavage with the FI*R*E procedure™ were about three times lower compared to those of the GC-MS methods reported previously by Landin et al. (LOD: 2 pmol/g globin) (Landin et al. 1996).

A general problem of Hb adduct quantification is that the cleavage efficiency of the isothiocyanate-mediated Edman degradation is unknown, leading to a method-inherent underestimation of Hb adduct levels. To improve the accuracy of the quantification, Monien et al. synthesized the peptide *N*-(2,3-dihydroxypropyl)-Val-Leu-anilide (DHP-Val-Leu-An), which was used to determine the Edman reaction turnover in separate samples. The average Edman efficiency for the cleavage of DHP-Val from DHP-Val-Leu-An in nine independent experiments was between 67.0% and 85.3% (mean ± SD = 77.7 ± 4.3%). The application of these values to the correction of DHP-Val levels determined in the set of regular erythrocyte samples analyzed on the same day improved greatly the accuracy of the quantification by isotope-dilution UHPLC-MS/MS (Monien et al. 2020).

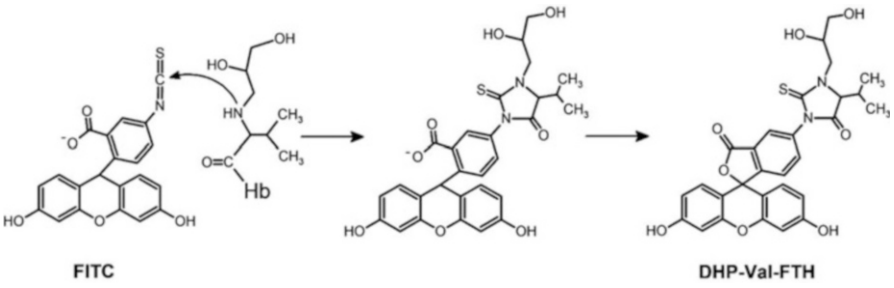

Fig. 2 Edman degradation of DHP-Val with the FI*R*E procedure™. The adduct DHP-Val in Hb is cleaved by a modified Edman degradation using fluorescein-5-isothiocyanate (FITC) to yield *N*-(2,3-dihydroxypropyl)-valine fluorescein thiohydantoin (DHP-Val-FTH), which is quantified by isotope-dilution UHPLC-MS/MS

Applications of DHP-Val as Biomarker of Glycidol Exposure

DHP-Val in Blood Samples from Rats and Humans

The adduct was first described by Landin et al. (1996), who observed that DHP-Val levels were 50% higher in blood samples of rats fed with fried animal feed compared to those observed in the control group (Landin et al. 2000). Also, direct oral administration of a high dose of glycidol or ester-bound glycidol (50 mg/kg body weight) in rats was shown to lead to DHP-Val levels in the range of about 13 nmol/g globin (Appel et al. 2013). Honda et al. observed that application of four different doses of glycidol to male rats led to an approximately linear increase of DHP-Val concentrations in blood samples retrieved 24 h after administration. The slope of the linear regression was 0.055 pmol DHP-Val/g Hb per μg glycidol/kg body weight (Honda et al. 2014).

Landin et al. compared DHP-Val levels in different groups of nonsmokers, smokers, and industry worker exposed to epichlorohydrin, a potential precursor of DHP-Val in Hb (Landin et al. 1997). In three subgroups of nonsmokers, mean DHP-Val levels were 7.3 pmol/g Hb (German industry workers, $n = 8$), 6.8 pmol/g Hb (German office clerks from the same company as the industry workers, $n = 3$), and 2.1 pmol/g Hb (control group of Swedish adults, $n = 6$). The data did not support the hypothesis that DHP-Val results from occupational exposure to the reactive epichlorohydrin. Instead, Landin et al. stated later that DHP-Val may originate from dietary intake of glycidol and GE formed by heat-treatment of food (Landin et al. 2000). Higher mean DHP-Val levels were observed in blood samples from smokers, 21.1 pmol/g Hb (German industry workers, $n = 7$), 13.1 pmol/g Hb (German office clerks, $n = 8$), and 9.5 pmol/g Hb (Swedish adults, $n = 4$), indicating that the inhalation by cigarette smoke contributes significantly to the overall glycidol exposure (Landin et al. 1997).

Honda et al. investigated the formation of DHP-Val in nonsmokers consuming GE-rich refined cooking oil. The mean adduct levels in participants (7.3 pmol/g Hb, $n = 14$) exposed to a specific oil with a high GE content and in the control population without specified exposure to the indicated oil (6.9 pmol/g Hb, $n = 42$) were not statistically different. Due to missing data on consumption of the cooking oil in question and the resulting overall glycidol exposure of the study participants, the study of Honda et al. did not provide the basis for a clear interpretation (Honda et al. 2012).

In more recent studies, the FITC-mediated Edman degradation was used for the quantification of DHP-Val. In blood samples of 11 nonsmoking adults, Abraham et al. determined median levels of 4.0 (range: 3.2–5.1) pmol/g Hb ($n = 11$) (Abraham et al. 2019). In children ($n = 50$), Aasa et al. observed a mean DHP-Val level of 7.3 ± 2.5 (range: 4.420) pmol/g Hb. In the same study, the mean DHP-Val level of 23.4 (range: 18.1–31.4) pmol/g Hb observed in smoking adults ($n = 6$) was much higher compared to that of nonsmoking adults 10.3 ± 2.7 (range: 6.3–14.0) pmol/g Hb (Aasa et al. 2019).

DHP-Val in Cord and Maternal Blood: Prenatal Exposure to Glycidol

The technique of DHP-Val quantification was used by Monien et al. to clarify the question if unborn children are exposed by prenatal transfer of glycidol from the circulation of their mothers. For this sake, 100 paired cord and maternal blood samples from the Belgian birth cohort ENVIRONAGE (ENVIRonmental influence ON AGEing in early life (Janssen et al. 2017)) were analyzed. Median levels of DHP-Val were 5.4 (range: 2.3–29.2) and 1.6 (range: < LOD–8.9) pmol/g Hb in samples of mother and newborns, respectively. In blood samples of mothers who smoked during pregnancy and in the cord blood samples of their newborns ($n = 6$), the median DHP-Val levels were 16.7 (range: 6.4–29.2) and 6.2 (range: < LOD–8.6) pmol/g Hb, respectively. The Spearman correlation coefficient of DHP-Val levels in cord and maternal blood samples was 0.63 ($p < 0.001$) among all mother-newborn pairs and 0.59 ($p < 0.001$) among mother-newborn pairs of nonsmoking mothers. The median ratio of DHP-Val levels of cord to maternal blood was 0.35 (range: 0.19–1.14). The data indicated that unborn children are exposed to glycidol due to a relatively unhindered passive transfer through the placental barrier (Monien et al. 2020).

Estimation of External Glycidol Exposure Using DHP-Val (*Reverse Dosimetry*)

Most of the studies on DHP-Val in humans focused on identifying influencing factors of glycidol exposure in different population groups, for example, smoking (in adults) or transplacental exposure (in unborn infants). The studies allowed drawing conclusions about the relative exposure, without permitting an estimation of the actual glycidol intake. However, the external dietary exposure to GE/glycidol is required to improve the risk assessment. Reverse dosimetry is the discipline to approximate external exposure from biomarker data (Clewell et al. 2008; Skipper and Tannenbaum 1990). Using a kinetic model, it is possible to estimate the mean exposure to a reactive substance from a single measurement of a specific Hb adduct assuming that a continuous exposure leads to a steady-state level of the adduct and that the adduct is chemically stable (Fennell et al. 1992). Under these circumstances, the daily dose (D) on a body weight basis can be calculated as follows:

$$D = \frac{2}{\tau \cdot k} \cdot H$$

with H as the adduct level (pmol/g Hb), τ as the lifetime of the adduct, and k, a proportionality constant (daily increase of adduct level per dose on a body weight basis). For the case of glycidol and DHP-Val formation, the constant k was determined in a recent study at the Federal Institute for Risk Assessment (BfR), in which 11 participants took part in a controlled intervention with a commercially available palm fat containing a relatively high amount of ester-bound glycidol

(8.7 mg glycidol/kg) over 28 days. The consumption of a daily portion of 36 g fat (individual glycidol doses between 2.7 and 5.2 μg/kg body weight per day) led to an increase in the mean adduct levels from 4.0 pmol DHP-Val/g Hb before the start of the intervention to 12.2 pmol DHP-Val/g Hb after 28 days. Fitting of the data with the model described by Fennel et al. (1992) allowed determining the mean value of k (0.082 pmol DHP-Val/g Hb per μg glycidol/kg body weight) and the mean adduct lifetime τ (104 days), which corresponds to the estimated lifetime of erythrocytes ($\sim 110 \pm 21$ days) (Bentley et al. 1974). The value of k derived in this study is valid for an oral exposure to GE, but is expected to be different in case of inhalational exposure to glycidol.

With the value of the adduct level per dose ratio k at hand, the external dietary glycidol exposure was calculated from the mean DHP-Val background observed in the 11 study participants (4.0 pmol/g Hb) to be 0.94 μg/kg body weight (Abraham et al. 2019). This value exceeds significantly the median value of the mean chronic exposure to glycidol from GE across dietary surveys estimated for the adult population in Europe by the EFSA (0.2 μg glycidol/kg body weight) (European Food Safety Authority 2016).

Also Aasa et al. used the value of k to calculate the mean glycidol exposure from mean DHP-Val levels in adolescents (7.3 pmol/g Hb, $n = 50$, 2014) and in nonsmoking (10.3 pmol/g Hb, $n = 6$, 1997) and smoking adults (23.4 pmol/g Hb, $n = 6$, 1997). The estimated mean dose levels of adolescents (1.4 μg glycidol/kg body weight), nonsmoking adults (2.0 μg glycidol/kg body weight), and smoking adults (4.9 μg glycidol/kg body weight) were even higher than those calculated for German adults by Abraham et al. (Aasa et al. 2019). It must be noted that the sampling time may be an influential parameter. The blood samples from German adults ($n = 11$) were from 2017 (Abraham et al. 2019), while those of Swedish adolescents and of nonsmoking and smoking adults were from 2014 and 1997, respectively. The effect of the mitigation strategies of GE formation in plant and vegetable oils studies in the last decade (Oey et al. 2019) on the human exposure may be monitored using DHP-Val as a biomarker.

Important Considerations for the Application of DHP-Val as Biomarker of Glycidol Exposure

Unknown Exposure Sources of Glycidol

The discrepancy between the external exposure values calculated from the mean background of DHP-Val in German adults ($n = 11$) (Abraham et al. 2019) or estimated using occurrence and consumption data of food in Europe (European Food Safety Authority 2016) may have different reasons. It is conceivable that there are oral or inhalational glycidol sources, which are currently not identified by the exposure estimation. Some foods with relevant levels of GE may not be identified yet, and there is hardly any data on the formation of GE by domestic food preparation procedures (Inagaki et al. 2016). In addition, the inhalational

exposure, for example, from passive smoking, was not considered by the dietary exposure estimate of the EFSA.

Biomarker Specificity: Other Xenobiotic Precursors of DHP-Val?

For the estimation of the dietary exposure to glycidol from the steady-state level of DHP-Val, it is also relevant to clarify the specificity of the biomarker: Is it possible that DHP-Val may be formed from reactive substances other than glycidol? Various compounds were considered as precursors for DHP-Val, that is, 3-monochloropropane-1,2-diol (3-MCPD), epichlorohydrin (Landin et al. 1997), 1-bromopropane (Ishidao et al. 2002), and allyl alcohol (Honda et al. 2012). However, none of these hypotheses were confirmed, and 3-MCPD and epichlorohydrin were shown to not contribute significantly to DHP-Val formation (Aasa et al. 2017; Ishidao et al. 2002; Landin et al. 1997; Wollin et al. 2014). In a recent study, Shimamura et al. administered relatively high single oral doses of glycidol, glycidol oleate, and the alternative precursors, 3-MCPD, epichlorohydrin, propylene oxide, 1-bromopropane, allyl alcohol, fructose, and glyceraldehyde dissolved in soybean oil by gavage in male mice and analyzed DHP-Val in blood samples 24 h afterwards. DHP-Val was detected (LOD 10 pmol/g Hb) only in mice treated with glycidol and glycidol oleate (Shimamura et al. 2020). In summary, there are no indications so far of reactive metabolites of xenobiotics other than glycidol that form the adduct DHP-Val in relevant amounts.

DHP-Val from Endogenous Exposure to Glycidol or Other Reactants?

A recent review summarized indications for endogenous sources contributing to the internal exposure to various heat-induced food contaminants independent from the dietary intake (Rietjens et al. 2022). For example, the mercapturic acid *N*-acetyl-S-(carbamoylethyl)-L-cysteine (AAMA), the main metabolite of the food carcinogen acrylamide, is not only formed as a consequence of external acrylamide exposure. In a group of 14 adults on an acrylamide free diet, Ruenz et al. observed high amounts of urinary AAMA after day 3 of the washout period, which were equivalent to a daily acrylamide exposure of 0.2–0.3 μg/kg body weight. Considering the EFSA estimation of the median adult exposure of acrylamide (0.4–0.6 μg/kg body weight), the authors followed that roughly one third of the AAMA excretion is not due to the dietary intake of acrylamide. To explain this, Ruenz et al. suggested that there is an endogenous exposure source of acrylamide (Ruenz et al. 2016). This raises the question whether there is another source of endogenous metabolites leading to the formation of DHP-Val. This was hypothesized first by Honda et al., however, without suggesting possible sources (Honda et al. 2012, 2014).

To answer the question whether DHP-Val formation occurs independently from the dietary glycidol uptake, we quantified DHP-Val levels in 27 nonsmoking adults with a conventional omnivore diet recruited in the "Risks and Benefits of a Vegan Diet" (RBVD) study (Weikert et al. 2020) and in 16 nonsmoking strict raw food

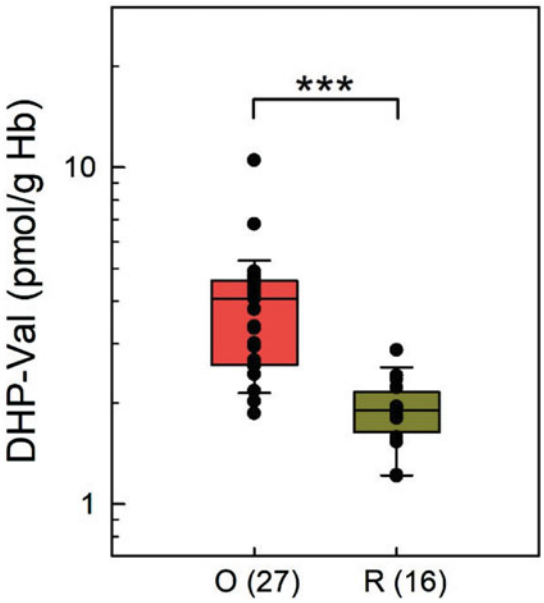

Fig. 3 DHP-Val in omnivores and strict raw food eaters. Levels of DHP-Val in blood samples from nonsmoking omnivores with conventional dietary habits including heated foodstuffs ($n = 27$, red box) and nonsmoking strict raw food eaters ($n = 16$, green box). Lines and boxes represent median values and the lower and upper quartiles, respectively, and the error bars represent the 10th and 90th percentiles ($^{***}p < 0.001$, Mann-Whitney rank-sum test)

eaters (Abraham et al. 2022), who did not consume any food heated to higher temperatures than 42 °C for at least 4 months (Fig. 3). The levels of DHP-Val in blood samples of strict raw food eaters (median 1.9, range 1.2–1.9 pmol/g Hb) were significantly lower ($p < 0.001$) compared to those of omnivores (median 4.1, range 1.9–10.5 pmol/g Hb). Comparison of the data suggests that under current conditions in Germany, about half of the DHP-Val levels in nonsmoking omnivores are formed independently from external exposure to glycidol and GE by the diet. Taking this into account, the median exposure to free and bound glycidol can be estimated to be 0.48 µg/kg body weight in the group of nonsmoking omnivores ($n = 27$). This is relatively close to the EFSA estimate of the dietary exposure to bound glycidol in adults (0.2 µg/kg body weight) (European Food Safety Authority 2016). However, as pointed out above, the EFSA estimate did not consider free glycidol in foodstuffs or GE and glycidol formed in the domestic food preparation.

Mini Dictionary

- **Glycidyl esters (GE)** are fatty acid esters formed during the refinement of vegetable oils. After ingestion, these oils and fats release glycidol in the gastrointestinal tract.
- **Glycidol** (2,3-epoxy-1-propanol) is an electrophilic, alkylating agent prone to react with cellular nucleophiles, such as DNA and proteins. It was classified as *probably carcinogenic to humans* (group 2A) by the International Agency for Research on Cancer (IARC).

- The **margin of exposure (MoE)** is the ratio between the lowest glycidol dose leading to a significant increase of tumor incidence in animals and the exposure estimates in humans.
- **Hemoglobin (Hb) adducts**, that is, the valine residues at the N-termini of Hb peptide chains modified by reactive compounds/metabolites, are used as biomarkers of medium-term exposure.
- ***N*-(2,3-dihydroxypropyl)-valine (DHP-Val)** is the adduct formed by the reaction of N-terminal valine residues in Hb with glycidol.
- The **Edman degradation** with isothiocyanates is used to cleave the modified valine adducts from the N-termini in Hb.
- **Ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS)** is a powerful analytical technique for the quantification of Hb adducts and other analytes using isotope-labeled standard compounds.
- **Reverse dosimetry** allows estimating of the external exposure to a toxicologically relevant substance from the quantitative information of a biomarker, for example, a metabolite excreted in the urine or a Hb adduct.

Key Facts of Valine Adducts in Hb

– Nitrogen atoms at the N-terminal valine residues in Hb are prone to react with electrophilic compounds.
– The modified valine residues are cleaved with isothiocyanates (Edman degradation) and the resulting thiohydantoin analytes are quantified using isotope-dilution mass spectrometry.
– Based on the Edman degradation, various biomonitoring methods for the determination of N-terminal adducts of acrylamide, ethylene oxide, epichlorohydrin, glycidol, glycidamide, benzyl chloride, and others were validated and are routinely used in occupational medicine.
– The valine adducts in Hb reflect the internal exposure to a reactive compound over a period of approximately 4 months corresponding to the lifetime of human erythrocytes in adults.
– A reverse dosimetry model was developed by Fennel et al. This allows calculating the daily external exposure to a reactive substance with a proportionality constant k, which describes the increase of the valine adduct level (pmol/g Hb) per dose (μg/kg body weight).
– The adduct per dose ratio k was determined in a controlled intervention study, monitoring the increase intervals of Hb adduct levels in a group of human participants exposed to defined amounts of ester-bound glycidol.

Summary

The exposure to the heat-induced food contaminant glycidol and its fatty acid esters (GE) leads to the formation of DHP-Val in Hb.

The absence of detectable DHP-Val amounts in blood samples of mice treated with alternative precursors, 3-MCPD, epichlorohydrin, propylene oxide, 1-bromopropane, allyl alcohol, fructose, and glyceraldehyde supported the notion that DHP-Val is a specific biomarker of glycidol exposure.

The quantification of DHP-Val levels in blood samples from different groups of study participants (mother/newborns, smokers/nonsmokers, omnivores/raw food eaters) allowed conclusions about the exposure sources of glycidol and GE.

Principal sources of glycidol exposure in adults are food (glycidol/GE) and tobacco smoke (glycidol), whereas unborn infants are exposed via placental transport of glycidol.

Using the adduct increase per dose ratio k (0.082 pmol DHP-Val/g Hb per μg glycidol/kg body weight) for reverse dosimetry, the mean oral exposure to glycidol was calculated for German adults (0.94 μg/kg body weight, $n = 11$) and Swedish adolescents (1.4 μg/kg body weight, $n = 50$).

The exposure data estimated from DHP-Val levels exceeded the median values of the mean chronic exposure to glycidol from GE across dietary surveys estimated for the adult population (0.2 μg glycidol/kg body weight) and for adolescents (0.3 μg glycidol/kg body weight) in Europe by the EFSA (European Food Safety Authority 2016).

The discrepancy between the results from reverse dosimetry calculation and estimations using occurrence and consumption data of food in Europe may be due to unknown sources of glycidol exposure not considered by EFSA, for example, yet unidentified foodstuffs with relevant levels of GE, the formation of GE by domestic food preparation procedures, and inhalational exposure of glycidol (e.g., from passive smoking).

The comparison of DHP-Val in blood samples from nonsmoking omnivores and raw food eaters suggests that about half of the DHP-Val levels in nonsmoking omnivores are formed independently from external exposure to glycidol and GE by the diet under current conditions in Germany.

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Biomarkers of Antibiotic Toxicity: A Focus on Metronidazole

8

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Abstract

Toxicity arising from the use of antibiotics is a limiting factor in health care. Individualized drug therapy has been useful in reducing the occurrence of antibiotic-induced toxicity but it has no quantitative or predictive importance. Biomarker-based strategies can be used to optimize patient-specific response to antibiotic therapy aimed at predicting and reducing antibiotic-induced toxicity. Metronidazole (MTZ), a nitroimidazole drug, is a classical antibiotic with wide application in human and veterinary medicine. Long-term use of MTZ is associated with neurotoxic effects amidst other forms of toxicity. Magnetic resonance imaging (MRI) changes with hyperintensities on T2-weighted or fluid-attenuated inversion recovery (FLAIR) sequences in the cerebellar dentate nuclei or splenium of the corpus callosum, and genotoxic indices such as sister chromatid exchange (SCE) and mitotic index (MI) in human lymphocytes are biomarkers of MTZ-induced toxicity.

Keywords

Neurotoxicity · Metronidazole · Magnetic resonance imaging · Genotoxicity · Neuropsychiatric · Encephalopathy · Ototoxicity · Cerebellar dysfunction · Sister chromatid exchange · Biomarker · DNA · Hepatotoxicity

Abbreviations

ALT	Alanine aminotransferase
ARG-1	Arginase-1
AST	Aspartate aminotransferase
B2M	Beta-2-microglobulin
CA	Chromosomal aberrations
CPK	Cell proliferation kinetics
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
DSB	Double-strand breakage
ELISA	Enzyme-linked immunosorbent assays
EMEA	European Medicines Agency
FDA	Food and Drug Administration
FLAIR	Fluid-attenuated inversion recovery
GFAP	Glial fibrillary acidic protein
GLDH	Glutamate dehydrogenase
GST-α	Glutathione *S*-transferase
HMGB-1	High-mobility group box-1
IARC	International Agency for Research on Cancer
IGFBP7	Insulin-like growth factor binding protein 7
L-FABP	Liver-type fatty-acid-binding protein
MAP-2	Microtubule-associated protein-2
MBP	Myelin basic protein

MCP-1	Monocyte chemotactic protein-1
MI	Mitotic index
MRI	Magnetic resonance imaging
MTZ	Metronidazole
NGAL	Neutrophil gelatinase-associated lipocalin
OCT	Ornithine carbamoyltransferase
PET	Positron emission tomography
PFOR	Pyruvate: ferredoxin oxidoreductase
PNP	Purine nucleoside phosphorylase
PON-1	Paraoxonase/Arylesterase 1
Rdxa	Nitroreductase
RI	Replication index
SCE	Sister chromatid exchange
SDH	Sorbitol dehydrogenase
T2	Transverse relaxation time
TIMP-2	Tissue inhibitor of metalloproteinases-2
TrxR	Thioredoxin reductase
UCH-L1	Ubiquitin C-terminal hydrolase L1
WHO	World Health Organization

Introduction

Antibiotics are one of the major groups of medicines used for maintenance of health worldwide. Their use has facilitated the treatment of diseases caused by pathogens (Hurkacz et al. 2021). Toxicity is a critical concern facing the use of many classes of antibiotics (Zagaria 2013; Mohsen et al. 2020). Several factors contribute to the occurrence of antibiotic-induced toxicities including indiscriminate use, as well as patient-, drug-, and disease-related factors among many others (Aulin et al. 2021). The concept of individualized drug therapy has been employed to limit the occurrence of drug-induced injury and promote drug safety (De Waele et al. 2014). This concept has greatly reduced the occurrence of antibiotic-induced toxicity, though it is not enough to quantify or foretell the occurrence of toxicity. Understanding human diversity and the interaction of antibiotics with various organs can promote the safe use of antibiotics. The emergence of biomarker-based strategies presents an opportunity to optimize patient-specific response to antibiotic therapy aimed at predicting and reducing antibiotic-induced toxicity (Aulin et al. 2021). However, application of this strategy in clinical practice is still slow as a result of knowledge gaps in terms of drug-patient-disease interaction (Belgrader et al. 1998). Several general organ toxicity biomarkers exist in clinical practice. Identification of patient-linked antibiotic-induced toxicity biomarkers would present a clearer approach to maximize drug therapy with limited toxicity (Griffin et al. 2019). Quantification of antibiotic exposure and changes in toxicity biomarker levels tend to ensure optimum therapeutic outcomes (Aulin et al. 2021).

Overview of Metronidazole

Metronidazole (MTZ) is a nitroimidazole drug developed over six decades ago for the treatment of trichomoniasis (Cosar and Julou 1959). Soon afterward its effectiveness against microaerophilic infections caused by *Giardia lamblia* (Schneider 1961) and *Entamoeba histolytica* (Powell et al. 1966) was demonstrated. MTZ is active against many other anaerobic and microaerophilic bacteria such as *Clostridium spp*. (Freeman et al. 1968; Ahmed et al. 1995; Chin and Hughes 2018), *Fusobacterium fusiforme* (Füzi and Csukás 1969), *Bacteroides fragilis* (Nastro and Finegold 1972), and *Helicobacter pylori* (Hirschl et al. 1988; Kim et al. 2007). MTZ is also effective against *Balantidium coli*, *Campylobacter spp, Gadnerella vaginalis*, and *Desulfovibria spp* (Leitsch 2019). Due to its relatively low cost and availability in oral, intravenous, and topical forms, as well as its rapid antibacterial effectiveness, MTZ is considered to be the gold standard for anaerobic infections, and forms a cornerstone of antibacterial therapy regimens in the WHO essential drug list (Leitsch 2019). MTZ has a wide range of applications in both human and veterinary practice including surgical prophylaxis.

Mode of Action

In contrast to most other antimicrobials, MTZ exhibits pleiotropism in its mode of action and reacts with a large number of molecules (Müller and Gorrell 1983). Importantly, MTZ, which strictly speaking is a prodrug, needs to be reduced at its nitro-radical group in order to become toxic to susceptible organisms (Edwards 1993). Several nitro-intermediates including oxamic acid and acetamide are generated during its metabolism; however, the cytotoxic intermediates are yet to be identified as they are rather unstable (Church et al. 1988; Dingsdag and Hunter 2018; Leitsch 2019) and the formation of these two derivatives does not account for all nitrogen atoms in the parent molecule. Reduction of MTZ takes place under anaerobic or microaerophillic conditions involving both enzymatic and non-enzymatic pathways (Willson and Searle 1975; Leitsch et al. 2009, 2011). There are distinct states of reduction involving electron transfer to the nitro group including the nitro-radical anionic state (transfer of one electron) (Lindmark and Müller 1976; Edwards 1993; Kulda 1999), the two-electron (nitroso) and the four-electron (nitroxyl) states (Wardman 1985).

In general, the first step in the mechanism is the reduction of the nitro group of the drug to the corresponding nitro-anion radical, other processes include redox recycling, formation of reactive radical species, and formation of nitroso, hydronitroxide, and amine compounds (Reveles et al. 2014; Lessa et al. 2015; Ceruelos et al. 2019). Several enzymes suggested to be involved in the reductive activation of MTZ include the pyruvate: ferredoxin oxidoreductase (PFOR) which catalyzes electron transfer via its iron-sulfur clusters resulting in the generation of nitro-radical anion (Lindmark and Müller 1976; Moreno et al. 1984; Chapman et al. 1985). Other enzymes thought to generate nitro-radical anions in vitro include the

cytochrome *P-450* reductase, xanthine oxidase, aldehyde oxidase, and ascorbate (Moreno et al. 1984; Ramakrishna and Ronald 1987). The nitro-anions thus produced can be further reduced under hypoxic conditions to form their corresponding nitroso, hydronitroxide, and amine compounds (Ramakrishna and Ronald 1987). Several flavin-dependent enzymes including thioredoxin reductase (TrxR) (Leitsch et al. 2007, 2009) and nitroreductase (Rdxa) (Olekhnovich et al. 2009) have also been described in the reduction of MTZ. The nitro-derivatives produced from the reduction of MTZ form thiol adducts which react with nucleotides (Ludlum et al. 1988) and cysteine (Leitsch et al. 2007), giving rise to GC-CG transversions leading to single and double DNA strand breaks, especially in AT clusters (Talapatra et al. 2010; Ceruelos et al. 2019). Degradation of translation elongation factor 1-γ (Leitsch et al. 2012) has also been described for the reductive derivatives of MTZ, all these effects put together may be responsible for its cytotoxicity.

Pharmacokinetics and Toxicity

MTZ is orally active with almost complete absorption and over 90% bioavailability; absorption is unaffected by infection (Ralph et al. 1974). Rectal and intravaginal absorption are 67–82%, and 20–56%, of the dose, respectively (Bergan et al. 1984). MTZ is distributed widely and has low protein binding (<20%) with the volume of distribution at steady state in adults ranging between 0.51 and 1.1 L/kg (Gulaid et al. 1978; Houghton et al. 1979). MTZ reaches 60–100% of plasma concentrations in most tissues studied, including the central nervous system, but does not reach high concentrations in placental tissue (Mattila et al. 1983). Clinical concentrations of MTZ seen in human plasma after a 2 g, one-time oral dose, commonly used to treat *T. vaginalis,* are approximately 300 μM; whereas peak concentrations of MTZ reached in human plasma after twice daily dosing with 500 mg (e.g., regimens to treat bacterial vaginosis) do not exceed 100 μM (Wang et al. 2011). MTZ is extensively metabolized by the liver to several metabolites. The hydroxy metabolite has biological activity of 30–65% and a longer elimination half-life than the parent compound (Somogyi et al. 1983). The majority of MTZ and its metabolites are excreted in urine and feces, with less than 12% excreted unchanged in urine (Gulaid et al. 1978; Houghton et al. 1979; Somogyi et al. 1983). The pharmacokinetics of MTZ is affected by acute or chronic renal failure, hemodialysis, continuous ambulatory peritoneal dialysis, age, pregnancy, or enteric disease (Somogyi et al. 1983; Houghton et al. 1985).

Although MTZ is generally well tolerated, its adverse effects are predominantly mild gastrointestinal disturbances such as nausea, abdominal pain, and diarrhea (Chin and Hughes 2018). Several neuropsychiatric effects (encephalopathy, cerebellar dysfunction, and seizures) as well as other side effects such as vertigo, impaired sleep, dizziness, and states of confusion, excitation, and depression (Ahmed et al. 1995) have been reported when the drug is used between 1 week and 12 weeks at doses ranging between 1 g and 2 g per day in humans and 40 mg/kg in animals (Kim et al. 2007). Apart from neurotoxicities, other adverse effects of

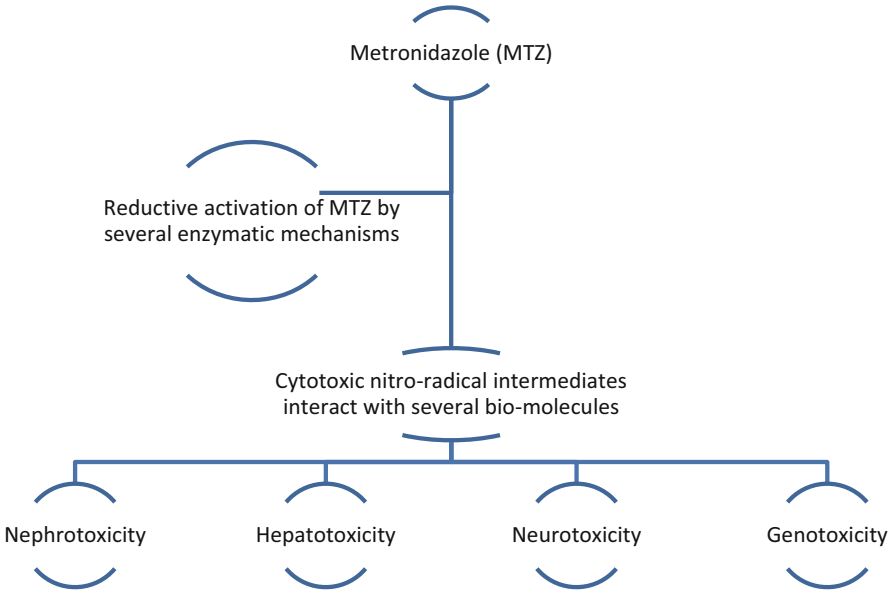

Fig. 1 Schematic representation of MTZ-induced toxicity

MTZ have been reported. These include DNA damage in human lymphocytes (Roe 1983; Celik and Ates 2006), bone marrow suppression (El-Nahas and El-Ashmawy 2004), immunosuppression in human blood lymphocytes (Mohammad et al. 2008), few reported cases of ototoxicity (O'Donnell and Barker 2016), and delayed immune-allergic hepatocellular liver injury (Kancherla et al. 2013). MTZ-induced toxicity seems to be due to the intermediates produced during its metabolism (Fig. 1) as it is a prodrug itself.

Biomarkers of Antibiotic-Induced Toxicity

Antibiotic-induced toxicities have become a major concern affecting different classes of medicines in the treatment of infectious diseases (Zagaria 2013) and these toxicities occur via various mechanisms (Fig. 2). These antibiotic-induced toxicities can present on short-term or long-term basis. The acute toxic effects of antibiotics are often reversible on discontinuation of the offending agent. However, they may result in treatment failure owing to abrupt discontinuation of therapy and this contributes to antibiotic resistance. Long-term toxicities often result in permanent damage and are often detected after chronic administration of the offending agent (Aulin et al. 2021), howbeit some long-term effects manifest early in the course of treatment. Quantification of antibiotic-induced toxicity is mostly not feasible in patients as it relies basically on histological findings which are sometimes postmortem.

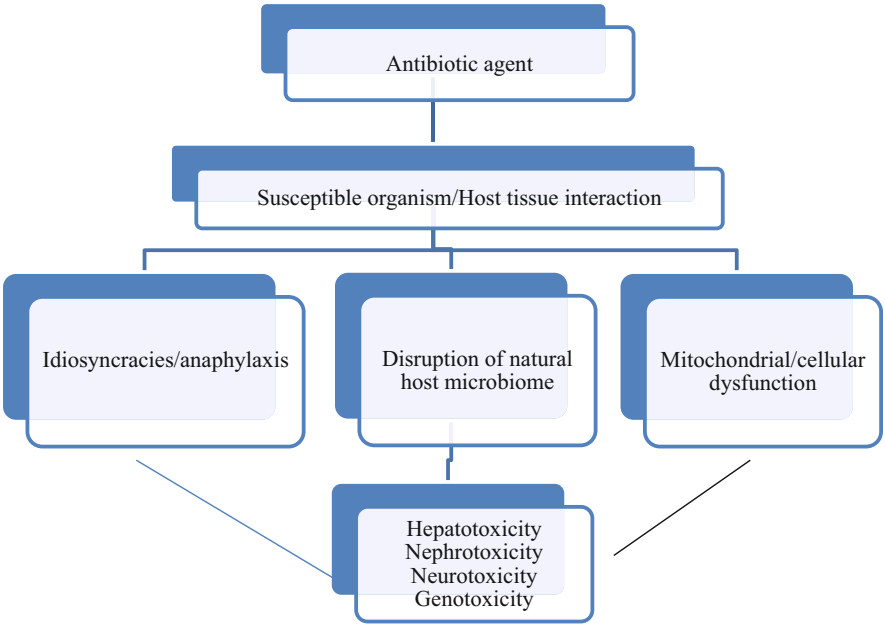

Fig. 2 Mechanisms of antibiotic-induced toxicities

Antibiotic-induced toxicity biomarkers can be useful in both quantification and prediction of toxicity.

Several biomarkers are available in clinical practice for accessing organ function. Prediction of antibiotic-induced toxicity may require specialized biomarkers as the general biomarkers are insufficiently specific and sensitive (Tajima et al. 2019). Biomarkers may be classified as predictive or prognostic (Atkinson et al. 2001; De Gruttola et al. 2001). Predictive biomarkers are useful tools in stratification of patients with tendency to develop antibiotic-induced toxicity on the premise of biomarker availability or absence. The availability of a specific toxicity biomarker in a patient can be used to single out high-risk patients and hence avoid toxicity as sensitivity and specificity are strictly adhered to (Dupuy et al. 2013). Biomarkers which can identify early signs of toxicity are invaluable in optimizing antibiotic therapy allowing for dosage adjustment where necessary (Tajima et al. 2019). A major setback in the use of biomarkers to maximize antibiotic therapy is the disparity in their measurement, interpretation, and translation of data which are mainly obtained from animal studies to humans.

Biomarkers of Nephrotoxicity

Nephrotoxicity is associated with a number of antibiotics. There are several traditional biomarkers of kidney function, such as serum creatinine and blood

urea nitrogen which lack specificity and sensitivity; these traditional markers are late indicators of kidney toxicity as they often rise after some loss of kidney function (Bonventre et al. 2010). A number of blood and urinary biomarkers including beta-2-microglobulin (B2M) produced by activated lymphocytes (Gautier et al. 2014); kidney injury molecule-1 (KIM-1), an epithelial cell adhesion molecule found in the proximal tubules, monocyte chemotactic protein-1 (MCP-1), and cystatin C, a component of all nucleated cells, have been identified as biomarkers for early antibiotic-induced kidney damage (Ozer et al. 2010; Vaidya et al. 2010). Neutrophil gelatinase-associated lipocalin (NGAL) and liver-type fatty-acid-binding protein (L-FABP) are emerging early nephrotoxicity biomarkers (Table 1) requiring further human studies (Griffin et al. 2019). Clusterin, a glycosylated protein commonly found in the kidney, is also an early nephrotoxicity marker secreted in urine, although there is a paucity of information regarding its usefulness in human studies (Rosenberg and Silkensen 1995). Out of these nephrotoxicity biomarkers, KIM-1, B2M, clusterin, and cystatin C are accepted by the Food and Drug Administration (FDA) and European Medicines Agency (EMEA) as highly sensitive and specific urinary biomarkers (Griffin et al. 2019). There is ongoing research to finding more specific and sensitive nephrotoxicity biomarkers with universal acceptability. Some promising molecules include interleukin-18 that demonstrates urinary increase at least a day before increase in serum creatinine levels (Edelstein 2017) and insulin-like growth factor binding protein 7 (IGFBP7), a marker of apoptosis in combination with tissue inhibitor of metalloproteinases-2 (TIMP-2), which are also recognized by the FDA howbeit with limited applicability (Vijayan et al. 2016).

Various antibiotics have negative effects on the kidneys, the aminoglycosides and polymyxins have known nephrotoxic effects. So far there are few reports of nephrotoxicity in animals linked to MTZ (Somogyi et al. 1983) with no reports from human data, although the histological results do not conform with the biomarker data from these studies. In summary, there are no specific biomarkers of MTZ-induced nephrotoxicity as there is a paucity of information in that regard.

Table 1 Markers of antibiotic-induced kidney toxicity

Regular markers	Predictive/Emerging biomarkers
Total protein	Beta-2-microglobulin (B2M)
Bilirubin	Kidney injury molecule-1 (KIM-1)
Creatinine	Monocyte chemotactic protein-1 (MCP-1)
Blood urea/Nitrogen	Cystatin C
Albumin	Neutrophil gelatinase-associated lipocalin (NGAL)
Kidney biopsy	Liver-type fatty-acid-binding protein (L-FABP)
CT scan	Clusterin
	Interleukin-18
	Insulin-like growth factor binding protein 7 (IGFBP7)
	Tissue inhibitor of metalloproteinases-2 (TIMP-2)

Hepatotoxicity Biomarkers

Hepatotoxicity is a major concern in therapeutics. Several classes of antibiotics are known to cause antibiotic-induced liver damage which may sometimes be fatal or result in severe hepatic damage. The liver enzymes (alanine [ALT] and aspartate [AST] aminotransferases) are traditional markers of liver injury, they lack the specificity and sensitivity required of predictive biomarkers of antibiotic-induced toxicity (Campion et al. 2013). Other factors like diet, exercise, and disease condition affect their serum concentrations. Changes in their serum concentrations also occur after some damage has occurred in the liver and sometimes these changes may not correlate with the histology of the liver (Bonventre et al. 2010). Glutamate dehydrogenase (GLDH), high-mobility group box 1 (HMGB-1), keratin-18 (k18), microRNA-122, and ornithine carbamoyltransferase (OCT) are more sensitive markers of hepatotoxicity compared to the traditional markers (Table 2) including the blood levels of aminotransferases and total bilirubin (O'Brien et al. 2002; Campion et al. 2013). Other markers of hepatic damage include paraoxonase/arylesterase 1 (PON-1), purine nucleoside phosphorylase (PNP), arginase 1 (ARG-1), sorbitol dehydrogenase (SDH), and glutathione *S*-transferase (GST-α). In addition, current research in proteomic technologies may lead to discovery of more sensitive biomarkers.

MTZ is not a known hepatotoxicant despite being extensively metabolized by the liver CYP2A6. Animal studies show variable reports with some changes in liver enzymes without histocompatibility. Most effects of MTZ on the liver are mild and reversible on withdrawal of the drug. Data from animal studies mostly do not translate to human effects although a few reports of MTZ-induced hepatotoxicity do exist (Kancherla et al. 2013). Despite the fact that antibiotic-induced hepatic damage occurs from use of several classes of antibiotics with amoxicillin-clavulanate combination being the most reported (Chalasani et al. 2008), it is scarcely so for MTZ. Antibiotic-induced liver damage due to delayed idiosyncratic reactions is rare with complex causes including drug and host (genetic and non-genetic) factors. It is challenging to investigate because of its rarity, the lack of experimental models, the number of medications that might cause it, and challenges to diagnosis. There is currently no biomarker for MTZ-induced hepatotoxicity.

Table 2 List of antibiotic-induced hepatotoxicity markers

Traditional markers	Predictive/Emerging biomarkers
Alanine aminotransferase (ALT)	Glutamate dehydrogenase (GLDH)
Aspartate aminotransferase (AST)	High-mobility group box 1 (HMGB-1)
Alkaline phosphatase (ALP)	Keratin-18 (k18)
Albumin	MicroRNA-122
Protein	Ornithine carbamoyltransferase (OCT)
Gamma-glutamyltransferase	Paraoxonase/Arylesterase 1 (PON-1)
Lactate dehydrogenase	Purine nucleoside phosphorylase (PNP) Arginase (ARG-1)
Prothrombin time	Sorbitol dehydrogenase (SDH)
	Glutathione *S*-transferase (GST-α)

Neurotoxicity Biomarkers

Neurotoxicity has been linked to a number of common antibiotics but diagnosis and prediction is a major challenge. Traditional means of identifying neurotoxicity involves the use of complex functional assessments, such as behavioral changes and electrophysiological measures, including histopathological assessment of neural tissues by the hematoxylin/eosin staining methods (Bolon et al. 2013). These methods lack the sensitivity and specificity expected from biomarkers. Biological fluid–based markers and emerging imaging technologies (Table 3) from minimally invasive techniques can help diagnose and predict neurotoxicity with wide applicability that is relevant across animal models and can be translated to clinical data. Some biofluid-based markers of neurotoxicity include microRNAs, F2-isoprostanes, translocator protein, glial fibrillary acidic protein (GFAP), ubiquitin C-terminal hydrolase L1 (UCH L1), myelin basic protein (MBP), microtubule-associated protein-2 (MAP-2), and total tau (Roberts et al. 2015). Enzyme-linked immunosorbent assays (ELISA) are already available for most of these biomarkers although they are not specific for antibiotic-induced neurotoxicity as same markers are present in most neurodegenerative and neuropsychiatric conditions (Rosen and Zetterberg 2013). A major setback is that acquisition of some of these biomarkers such as those in cerebrospinal fluid (CSF) requires invasive sampling. Neuroimaging techniques such as magnetic resonance imaging (MRI) and positron emission tomography (PET) also serve as biomarkers and have the advantage of being minimally invasive compared to biomarkers from the CSF (Hanig et al. 2014). A combination of imaging techniques and fluid-based markers would provide both diagnostic and predictive biomarkers of antibiotic-induced neurotoxicity.

The most worrisome adverse effect of MTZ is neurotoxicity which includes both central and peripheral neurotoxicities. MRI is the mainstay of predicting and diagnosing MTZ-induced neurotoxicities. This technique can predict cellular toxicity as it detects alterations in tissue characteristics such as cellular integrity, cell density, and water redistribution in vivo (Roberts et al. 2015). The most useful MRI technique measures T2 relaxation since it is simple and can provide evenly distributed

Table 3 List of traditional and predictive biomarkers of antibiotic-induced neurotoxicity

Traditional markers	Predictive biomarkers	MTZ toxicity biomarkers
Motor activity tests Behavioral tests Electroencephalogram Nerve conduction velocity	microRNAs F2-isoprostanes Translocator protein Glial fibrillary acidic protein (GFAP) Ubiquitin C-terminal hydrolase L1 (UCH L1) Myelin basic protein (MBP) Microtubule-associated protein-2 (MAP-2) Total tau	Magnetic resonance imaging T2-weighted and fluid-attenuated inversion recovery (FLAIR) hyperintensities

Table 4 Antibiotic-induced genotoxicity markers

Traditional markers	Emerging biomarkers	MTZ toxicity biomarkers
Comet assay Bacterial Ames test Alkaline unwinding Hydroxyapatite chromatography	Replication index (RI) Micronuclei (MN) Chromosomal aberrations (CA) DNA strand breaks (DSB)	Sister chromatid exchanges (SCEs) Mitotic index (MI)

time course scans and quantitative metrics (Hanig et al. 2014). The biomarkers of MTZ-induced neurotoxicity are typical bilateral or symmetrical imaging changes with hyperintensities on T2-weighted or fluid-attenuated inversion recovery (FLAIR) sequences in the cerebellar dentate nuclei or splenium of the corpus callosum (Kim et al. 2007; Sørensen et al. 2020), being the most affected regions consistent with all MTZ-induced neurotoxic events.

Genotoxicity Biomarkers

Long-term use of antibiotics with genotoxic effects may cause mutations and consequently cancers but from clinical data most antibiotics with genotoxic effects in animal studies are not carcinogenic in humans. MTZ is thought to exert its antibacterial effect by causing DNA damage in susceptible organisms (Talapatra et al. 2010; Ceruelos et al. 2019), and genotoxicity in human lymphocytes has been reported as one of its adverse effects (Roe 1983; Celik and Ates 2006). MTZ is reported to alter the frequency of SCEs in peripheral blood lymphocytes and MI of the black-stripped capuchin (Mudrya et al. 2011). However, mutagenicity and carcinogenicity of MTZ in humans is a matter of current debate as many conflicting reports exist in literature (Konopacka et al. 1990; Buschini et al. 2009; Mudrya et al. 2011), most of which are animal data with a few on human lymphocytes (Roe 1983; Celik and Ates 2006). Even with these conflicting data the International Agency for Research on Cancer (IARC) classifies it as carcinogenic (Brambilla et al. 2012). Traditional methods of identifying genotoxicity (Table 4) are available but they are devoid of predictive capacity. Some biomarkers of antibiotic-induced genotoxicity include mitotic index (MI), cell proliferation kinetics (CPK) measured as replication index (RI), sister chromatid exchanges (SCEs), micronuclei (MN), chromosomal aberrations (CA), and DNA strand breaks (DSBs) (Mudrya et al. 2011). The most reported biomarker of MTZ-induced genotoxicity is SCE and reduced MI in human lymphocytes.

Application to Prognosis

MTZ is a widely used antibiotic with clinical efficacy across all age groups and gender. So far toxicities have been reported in adults and some geriatric patients (Chin and Hughes 2018). No organ toxicity has been reported in children apart from

the regular side effects, although it could be due to underreporting. The biomarkers identified for MTZ-induced toxicities could be studied and applied safely in children receiving long-term treatment with MTZ as MRI scans are devoid of X-rays (Zhang et al. 2019; Zagaria 2013).

Mini-Dictionary of Terms

- **Genotoxicity**: alteration of DNA or other genetic materials by a toxicant.
- **Hepatotoxicant**: a substance which when introduced into the body has the potential to harm the liver.
- **Invasive sampling**: collection of biological samples requiring puncturing of skin or other tissues with medical equipment.
- **Microaerophilic**: conditions requiring minute quantities of free oxygen.
- **Pleiotropism**: a phenomenon in which an active drug molecule has multiple mechanisms of action by interacting with several biological molecules.

Key Facts of Antibiotic-Induced Toxicity

- Long-term high-dose use of antibiotics can result in organ damage which may be irreversible.
- Individualized therapy or therapeutic drug monitoring is key in reducing antibiotic-induced toxicity.
- Individualized therapy is not sensitive enough to predict or prevent antibiotic-induced toxicities.
- Identification and incorporation of biomarker-based treatment guidelines into clinical practice are key to predicting, quantifying, and preventing antibiotic-induced toxicity.
- A knowledge gap exists between the availability and functionality of biomarkers from animal studies and their application in humans.

Summary Points

- Antibiotic-induced toxicities are largely unquantified and insufficiently reported.
- Fluid-based biological molecules can predict and diagnose persons prone to antibiotic-induced toxicity.
- The toxic effects of MTZ are mainly due to the formation of toxic anionic intermediates.
- Neurotoxicities and probably genotoxicity are the most prominent MTZ-induced toxicities.
- A combination of MRI and fluid-based biological molecules are the best biomarkers of MTZ-induced toxicity.

Conclusion

Toxicity is a serious complication and limiting factor to maximizing antibiotic therapy. Institution and incorporation of biomarker-based treatment guidelines in clinical practice will go a long way in mitigating organ injury due to antibiotic use. A key factor that will aid this process is the harmonization of baseline data originating from the large number of biomarkers already in existence, as well as their interpretation and translation into clinical practice. Several biomarkers may need to be combined to give definitive prediction or diagnosis of antibiotic-induced toxicity. Further multisectorial research is required to bridge the knowledge gap that exists and also create easy-to-use cost-effective test kits to achieve the desired goal.

Cross-References

- ▶ Biomarkers of Neurotoxicity
- ▶ DNA Adducts as Biomarkers in Toxicology
- ▶ Drug-Induced Nephrotoxicity and Use of Biomarkers

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Part II

Panels and Arrays

Pro-inflammatory Markers of Environmental Toxicants

9

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Contents

Abstract

Achievement of accurate prognosis, prediction, and treatment of diseases in modern medicine is managed through detecting changes of crucial molecules, named biomarkers, with metabolic, signalling, and pro- or anti-inflammatory roles in body fluids and tissues, causing minimal discomfort for the patients. The present study is focused on potency and impact of persistent environmental toxicants on human health and their effects on blood biomarkers, which manifest themselves by the process of inflammation. Significantly toxic metabolites of

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polycyclic aromatic hydrocarbons (PAHs), as pollutants, also cause liver damage and ROS buildup which is the main reason for activation of pro-inflammatory pathways. Long-term PAH exposure may lead to (1) cardiovascular disorders (including ischemic stroke, myocardial infarction, and atrial fibrillation), (2) pneumonia, (3) cancer, and (4) hepatic disorders. These disorders are characterized by oxidative stress and inflammatory response leading to aberrant cellular responses, including cell death, tissue damage, and neoplasia. In this respect, 18 kDa translocator protein (TSPO) is known to be able to modulate oxidative stress, and inflammatory responses, as it is also known to be involved in cardiovascular disorders, brain disorders, lung disorders, cancer, and liver problems. TSPO is among the novel mitochondrial biomarkers connected with ROS formation and cellular integrity, by which it might be effective in diagnosing inflammation and response to therapy. In the present study, we found that TSPO may be used as a biomarker for hepatic injury, provoked by 7,12-dimethylbenz[a]anthracene (DMBA, one of PAHs) and of the curative effects of medicinal fungus in an animal model.

Keywords

PAHs · 7,12-dimethylbenz[a]anthracene (DMBA) · Inflammatory mechanisms · Toxic influence · ROS biomarkers · Hepatotoxicity · Antioxidant defense · 18 kDa translocator protein (TSPO) · Natural antioxidants · *Phellinus torulosus*

Abbreviations

^{18}F-FEDAC	N-benzyl-N-methyl-2-[7,8-dihydro-7-(2-[18F]fluoroethyl)-8-oxo-2-phenyl-9H-purin-9-yl]acetamide
2-Cl-MGV-1	2-(2-Chlorophenyl) quinazolin-4-yl dimethylcarbamate
^{3}H-PK11195	1-(2-Chlorophenyl)-N-[^{3}H]-methyl-N-(1-methylpropyl)-3-isoquinoline carboxamide
AhR	Aryl hydrocarbon receptor
ALP	Alkaline phosphatase
ALT	*Alanine aminotransferase*
AOPP	Advanced oxidation protein products
AST	*Aspartate aminotransferase*
BaP	Benzo(a)pyrene
BP	Binding potential
Cat	*Catalase*
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
DMBA	7,12-Dimethylbenz[a]anthracene
eNOS	*Endothelial nitric oxide synthase*
HDL	High-density lipoprotein
HWE	Hot water extract
IL-1β	Interleukin-1beta

IL-6	Interleukin-6
iNOS	*Inducible nitric oxide synthase*
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low-density lipoprotein
LPO	Lipid peroxidation
MPO	*Myeloperoxidase*
mPTP	Mitochondrial permeability transition pore
NO	Nitric oxide
PAHs	Polycyclic aromatic hydrocarbons
PCC	Protein carbonyl content
$PM_{2.5}$	Fine particulate matter
PON-1	*Paraoxonase-1*
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	*Superoxide dismutase*
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor alpha
TSPO	18 kDa translocator protein
UA	Uric acid
URAT1	Urate anion transporter 1

Introduction

Accurate prognosis, prediction, and treatment of diseases in modern medicine is achieved through detecting changes of crucial molecules, named biomarkers, with metabolic, signalling, and pro- or anti-inflammatory roles in body fluids and tissues, causing minimal discomfort for the patients (Califf 2018). Large numbers of studies and experiments have been focused on enlarging the list of "diagnostically valuable" molecules further verifying their correlation with clinical outcome and severity of the disease, as well as therapy efficiency (Aronson and Ferner 2017; Califf 2018; Ou et al. 2021). Validation is also an important yet difficult step toward the inclusion of biomarkers in clinical practice. However major breakthroughs have been achieved in this area by introducing randomized and blinded clinical trials, thus avoiding bias, and by establishing well-defined methods to ascertain their sensitivity, specificity, accuracy, and predictive value (Amur et al. 2015; Kraus 2018; Ou et al. 2021). Still, obtaining a significant number of "suitable molecules" for easier diagnosis and treatment of almost every pathology is the most important impact of biomarkers at present. In particular, biomarkers have facilitated the screening and prevention of diseases and conditions with high prevalence and incidence originating from ubiquitously present toxicants, resulting in the possibility of conducting clinical trials with routine noninvasive laboratory examinations such as urinalysis in patients exposed to different types of polycyclic aromatic hydrocarbons (PAHs) that offer a crystal clear image about the progression of metabolic diseases such as type 2 diabetes mellitus (Zhang et al. 2020).

Polycyclic Aromatic Hydrocarbons (PAHs): Toxic Effects and Organ Damage

Environmental pollutants with their ubiquitous existence in the biosphere and the different forms of these substances that can be dispersed among the human population are possibly part of the most potent toxicants which continuously run counter to healthy lifestyle and represent a serious threat to human health (Manisalidis et al. 2020).

PAHs formed during suboptimum combustion of organic materials from various sources constitute a major part of environmental pollutants predominantly found as fine particulate matter – $PM_{2.5}$ particles (83–90% of total PAHs) (Hassanvand et al. 2015; Låg et al. 2020). Other exposure may occur as a result of cigarette smoking, as well as consumption of contaminated food or water. In general, these hydrocarbons undergo biotransformation by the cytochrome P450 enzymes to metabolically active entities of diol epoxides and o-quinones which are capable of lipid peroxidation, protein modification, and DNA mutation (Jie et al. 2018).

The effects of PAHs on human health depend mainly on the length, dose, and route of exposure, including subjective factors such as pre-existing health and age. Likewise, acute exposure to these substances can cause mild irritation, vomiting, or nausea, but long-term exposure may lead to more severe disorders such as the three cardiovascular endpoints (ischemic stroke, myocardial infarction, and atrial fibrillation), pneumonia, or cancer (Nakano and Otsuki 2013; Adaji et al. 2019; Yazdi et al. 2021). Since the liver is a primary organ involved in biotransformation of food and drugs, hepatic disorders present a major concern worldwide (Låg et al. 2020). These disorders are not only caused by toxic chemicals and xenobiotics but also by immunosuppressant, anti-inflammatory, and antitubercular drugs (Shen et al. 2019). Newer research proved the significant impact of molecular biomarkers such as caspase-3, Beclin-1, and Bcl-2 expression in the detection of the severe mitochondrial damage caused by 7,12-dimethylbenz[a]anthracene (DMBA) exposure (Hosny et al. 2021).

Furthermore, the findings of our investigation presented in the sections below are focused on the interaction of novel biomarkers in order to assay levels of mitochondrial dysfunction derived from xenobiotics from human environment.

Effects of PAHs on Blood Biomarkers

Scientific confirmation about the potency and toxic impact of PAHs has led to inclusion of biomarkers which accurately reveal their deleterious effects and their alterations could be directly associated with therapy efficacy. As mentioned earlier, crucial target of DMBA damaging outcome is the liver, where it is actually metabolized. Elevated levels of serum biomarkers like alanine and aspartate aminotransferase (ALT and AST, respectively) and alkaline phosphatase (ALP) are often the primary indicators of liver failure accompanied by parenchymal damage, necrosis, and injury of the biliary epithelial cells independent of its etiology (Giannini et al. 2005; Fu et al. 2020). With reference

to PAH's hepatotoxicity, enhanced aminotransferase levels indicate ischemic or toxic liver injury in more than 90% of cases with acute hepatic injury. Moreover, due to hypoxic and toxic damage, it is important to stress that aminotransferase levels tend to decrease rapidly after peaking (Hamdy et al. 2016; Kim et al. 2019; Smiljevska-Ristovska et al. 2020).

The role of uric acid (UA) as a metabolic product is also debated, due the evidences of its dual nature as pro- and antioxidant molecule (Sautin and Johnson 2008). So far, it is clear that only acutely elevated or exogenously added UA can act as a radical scavenging, metal chelating molecule which participates in inhibition of protein nitrosylation and peroxidation, specifically in the central nervous system (CNS) (Sautin and Johnson 2008; Kang and Ha 2014). In contrast, significant elevations of serum UA can result in increased reactive oxygen species (ROS) production and activation of pro-inflammatory pathways in vascular endothelial and smooth muscle cells, thus establishing hyperuricemia as significant risk factor for metabolic syndrome and cardiovascular disease (Kang and Ha 2014). Hyperuricemia is a very common side effect of PAH exposure (Dimitrova-Shumkovska et al. 2010b; Mohamed et al. 2021) which according to the above might be a contributing factor for aggravation of the inflammatory response.

Recent studies have shed some new light into this perspective given the positive correlation shown between UA levels and pro-inflammatory molecules such as high-sensitivity C-reactive protein (CRP), fibrinogen, ferritin, and also complement C3 in nondiabetic subjects and in vitro evidences about UA involvement in activating NF-κB pathway (Spiga et al. 2017). In addition, it has been found that mature macrophages exposed to a moderate dose of UA (0.23 mM) are able to stimulate production of tumor necrosis factor alpha (TNF-α) via activation of the toll-like receptor 4 (TLR4) which also upregulates the NF-κB pro-inflammatory pathway. Furthermore, authors suggest the possible involvement of urate anion transporter 1 (URAT1) in the pro-inflammatory effects of UA since its blockage significantly decreased TNF-α production. However, when higher doses of UA were applied (0.45 and 0.9 mM), no changes in the TNF-α were recorded compared to unexposed cells suggesting saturation of URAT1 as possible explanation (Martínez-Reyes et al. 2020).

The Effect of Inflammatory Biomarkers

Pro-inflammatory biomarkers are a useful tool for determining the negative influence of PAH exposure, particularly the increase in ROS formation which is often accompanied by acute inflammatory response as a result of endothelial damage (Mittal et al. 2014). Significant elevations of conventional pro-inflammatory cytokines such as interleukin-1β (IL-1β), IL-6, and TNF-α were recorded in multiple reports, particularly after a successful lung or breast tumor induction by chronic exposure with DMBA (Wang and Zhang 2017; Kumar et al. 2021).

Inflammation is often associated with significant elevation of the vasodilator nitric oxide (NO), which is gaseous transmitter that contributes to the normal cellular

signalling in tissues produced by NO synthase. Injurious stimuli caused by the organic toxicant favor the increase of the inducible nitric oxide synthase (iNOS) which produces large quantities of this gaseous product in a short period of time and facilitates tissue damage, rather than the endothelial nitric oxide synthase (eNOS), which activity is mostly regulated by the vascular endothelial factor and shear stress (Iwakiri and Kim 2015; Zhang et al. 2016). Increases in NO levels are associated with the buildup of ROS and inflammatory cytokines as demonstrated by *in vivo* experiments (Ajayi et al. 2016; Eğimezer et al. 2020; Smiljevska-Ristovska et al. 2020). It has been established that NO has a dual function. Namely, it may lead to formation of highly reactive genotoxic species such as peroxynitrite and nitrosonium anion involved in pro-apoptotic pathways as a somewhat protective mechanism against carcinogenesis, but on the other hand, accumulation of these reactive nitrogen species (RNS) might provoke DNA damage, thus propagating cancer formation (Eğimezer et al. 2020).

Another protective mechanism against tissue injury, in order to inhibit subsequent infiltration of mononuclear cells in inflamed tissues provoked by increased vascular permeability, is elevation of myeloperoxidase (MPO) activity, an enzyme that eliminates the excess of hydrogen peroxide by converting it to hypochlorite (Klebanoff 2005; Khan et al. 2018). The connections made between increases in levels of lipid peroxidation (LPO), protein carbonylation, and MPO activity, specifically the generation of tyrosyl radical, nitrogen dioxide, and phenolic cross-links to proteins, place this enzyme at the center of inflammatory processes, thereby claiming its suitability as a biomarker (Davies 2011). Even though fluctuations in MPO activity have been detected in a large number of diseases including atherosclerosis, neurodegenerative diseases, diabetes, lung injury, and cancer (Khan et al. 2018), more research is needed to specify the effects of PAH on MPO levels. So far, indeed a positive correlation has been established between tissue MPO increases and the dose of applied polycyclic toxicants (Ajayi et al. 2016; Smiljevska-Ristovska et al. 2020).

Effects of PAH on Oxidative Stress and Mitochondrial Damage

As mentioned above, most common "symptoms" of exposure to environmental toxicants include increased formation of ROS disturbing the redox balance in cells by depleting reduced glutathione content and reducing activities of antioxidant enzymes. Oxidative damage caused directly by exposure to PM or their components induces inflammation which can ultimately lead to chronic conditions such as cardiovascular or lung diseases (Øvrevik et al. 2015; Holme et al. 2019). The stage of development of such conditions can be monitored via measurement of ROS generation. The final outcome can be assessed by quantifying the levels of well-established ROS biomarkers, such as lipid peroxidation products (LPO), protein carbonyl (PCC), and advanced oxidation protein products (AOPP) as well as GSH and antioxidant enzyme activities of catalase (Cat), as well as superoxide

dismutase (SOD) which can provide a fairly accurate image about the degree of tissue injury (Marrocco et al. 2017).

Scientific evidences also suggest the involvement of aryl hydrocarbon receptor (AhR) in mediating the inflammatory response by regulating expression of pro-inflammatory genes with xenobiotic response elements present in their promotor regions (Fardel 2013; Kim and Pjanic 2017) by triggering NF-κB signalling pathway (Vogel and Matsumura 2009) and by increasing the cytosolic calcium levels (Tomkiewicz et al. 2013). Recent studies also confirmed that the tumorigenic effects of DMBA are made possible by inhibiting apoptosis via upregulation of Bcl-2 and decrease in the expression of caspase-3, along with inhibition of autophagy by reducing the expression of Beclin-1 (Karabulut et al. 2014; Hosny et al. 2021) (Fig. 2). In light of these assertions, it can be stated that mitochondrial biomarkers might unveil new prospects in determination of the exact molecular mechanisms "responsible" for PAH-initiated suppression of autophagy and apoptosis.

18 kDa translocator protein, a benzodiazepine receptor localized on the outer mitochondrial membrane, is crucial for maintaining cellular homeostasis through its involvement in cholesterol transport, steroid synthesis, permeability transition pore opening, proliferation, and apoptosis by regulating mitochondrial ROS production, calcium efflux, and ATP synthesis (Papadopoulos et al. 2006; Kugler et al. 2008; Veenman et al. 2010). Studies also highlighted its role in regulation of nuclear gene expression, thereby participating in "cellular remodelling" by modifying protein synthesis (Yasin et al. 2017). Given its ubiquitous expression, 18 kDa translocator protein (TSPO) has been recognized not only for its potential as a diagnostically valuable biomarker for cancer and for brain injury and disease but also for its potential as a useful tool to assay levels of mitochondrial damage and inflammation derived from xenobiotics or PAHs mostly by its elevated binding potential (BP) (Veenman et al. 2015; Ghadery et al. 2017; Dimitrova-Shumkovska et al. 2020a); however such steroid and BP elevation may not be a universal response since decreases in BP were reported in non-steroidogenic cells after DMBA inflicted oxidative stress (Dimitrova-Shumkovska et al. 2010c).

Natural Antioxidants as Remedy Against PAH Toxicity

Recent studies were focused on evaluating potential neutralizing and/or inhibiting cytotoxic effects of natural antioxidants, supplemented in food or drinking water of animals exposed to potent carcinogens and environmental toxicants (Krishnamoorthy and Sankaran 2016; Horng et al. 2017). Discoveries about significant cancer preventive capabilities of dietary supplements (35% of cancer) and the increased number of surviving patients using natural antioxidants (up to 81%) have additionally promoted the use of dietary antioxidants. In addition, their efficacy depends on the dose and time of supplementation (Cassileth and Yarett 2012; Akanji et al. 2020; Dimitrova-Shumkovska et al. 2020b). Namely, decline of cytokines levels were dose dependent as established during treatment with active ingredients

from *Zingiber officinale* (6-gingerol) and the roots of the plant *Boerhavia diffusa* (boeravinone B) (Sun et al. 2021; Wang et al. 2021).

In terms of the confirmed antioxidant properties of medicinal mushrooms, regardless of their application as traditional remedies, several species with strong chelating and scavenging characteristics were also included in current medicine as safe and functional prophylactics suppressing diabetes, hypertension, inflammation, or cancer (Martel et al. 2017; Du et al. 2018; Ogidi et al. 2020).

Phellinus torulosus as a Prophylactic Against DMBA Hepatotoxicity

As a result of the ongoing extensive and thorough research regarding the bioactive properties of mushrooms, the number of analyzed species with potent antioxidant capacities and therapeutic properties is constantly increasing By the same token popularizing the consummation and use of *Lentinus edodes* (shiitake), *Grifola frondosa* (maitake), *Pleurotus ostreatus*, and *Ganoderma lucidum* as species known for their high nutritional and pharmaceutical values (Ogidi et al. 2020).

As a popular genus growing in the wild with confirmed medicinal properties, *Phellinus* spp. have been used for centuries in the Far East. Likewise, several studies evaluated beneficial effects of extracts from *Phellinus* spp. on glucose level in a diabetic animal model (Feng et al. 2018), inhibition of protein modification (Soler-Cantero et al. 2012; Kalogianni et al. 2020), as well as hepatoprotective effects against drug-induced liver fibrosis (Huang et al. 2018). Similar results were obtained by treatment with *P. igniarius* extracts of alcohol-induced liver injury (Dong et al. 2018). It is confirmed that most *Phellinus* species present antimicrobial, anti-inflammatory, and antidiabetic actions, while mentioned anticancer properties were established only for *P. linteus* (Azeem et al. 2018). *Phellinus torulosus* also known as *Fuscoporia torulosa* is another member of the wood-decay fungi with well-established in vitro antioxidant properties, but thus far little research has been carried out in order to reveal its in vivo antioxidant effects (Seephonkai et al. 2011; Azeem et al. 2018).

In order to search for a novel remedy for liver failure, instigated by the cytotoxic and inflammatory effects of PAHs elaborated earlier, we exposed 12-week-old female C57BL/6 wild-type mice with a single dose of 5 mg/kg DMBA administered by gavage, followed by daily oral administration of 0.5% hot water extract (HWE) in drinking water for a period of 10 weeks. To confirm the hepatotoxic effects of applied toxicant, as well to measure therapeutic effect of the fungal extract, oxidative stress markers (LPO, PCC, and GSH) were measured in liver tissue. The obtained data shows significantly increased lipid peroxidation (45.16% increase vs control mice, $p < 0.01$) and formation of protein carbonylation in liver of DMBA exposed mice (2.5 times higher value, $p < 0.01$, Table 1). The environmental toxicant provoked small but significant glutathione depletion (by 14.78% vs vehicle control, $p < 0.05$), which additionally increased levels of ROS formation, thus modulating mitochondrial functioning as reported by the fluctuations of TSPO. However,

Table 1 Oxidative stress parameters in liver tissue of control, DMBA-exposed, and DMBA-exposed + *P. torulosus*-administered mice

Parameter	Control group	DMBA exposed	DMBA exposed + *P. torulosus* administered
PCC (nmol/mg)	0.96 ± 0.17	2.49 ± 0.27**	1.71 ± 0.14$^{\#\#}$
TBARS (nmol/mg)	1.24 ± 0.12	1.80 ± 0.35**	1.48 ± 0.07
GSH (nmol/mg)	38.70 ± 4.70	32.98 ± 2.71*	37.39 ± 2.94

Data are presented as mean ± SD and analyzed with Mann–Whitney U test. Levels of statistical significance: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus vehicle control; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ versus DMBA exposed group

10-week supplementation with aqueous extract from *P. torulosus* did not significantly affect protein oxidation rate in liver tissue, but rather led to a decrease in lipid peroxide levels while restoring the levels of reduced glutathione.

These results confirm that *P. torulosus*, even in small doses, administered over a relatively long period of time, enhances the cellular defense mechanisms in liver tissue against the deleterious effects of the environmental toxicant DMBA.

P. torulosus Effects on TSPO Binding Potential

In view of its ubiquitous expression and modulation of its ligand binding potential (BP) as homeostatic responses, closely associated with mitochondrial ROS generation rates (Veenman et al. 2007; Bonsack and Sukumari-Ramesh 2018), we assayed TSPO to confirm the favorable results of *P. torulosus* scavenging and reducing abilities established by in vitro analysis (data not shown; unpublished data). Even though this receptor is mostly exploited as biomarker for neuro-inflammation and cancer (Iannaccone et al. 2013; Gerhard 2016; Dimitrova-Shumkovska et al. 2020a), appreciable TSPO levels have also been detected in rat's liver which enabled its use as marker for liver fibrosis and injury (Dimitrova-Shumkovska et al. 2010a; Hatori et al. 2015). TSPO expression was also targeted in hepatocytes, due to their tendency for structural alterations, cellular deterioration, and oxidative damage given the increase in the rates of metabolic activity after DMBA treatment (Wang et al. 2010).

In conformity with the results of increased oxidative damage in the DMBA exposed experimental group in our study, a significant elevation of TSPO density was established, together with higher affinity for its ligand ($p < 0.01$) (Table 2 and Fig. 1c, d). Given scientific evidence from previous studies, such as TSPO involvement in promoting mitochondrial ROS formation by modulating ATP synthase activity and cardiolipin peroxidation, as well as its participation in the mitochondrial apoptosis cascade via opening the mitochondrial permeability transition pore (mPTP) (Veenman and Gavish 2012; Veenman et al. 2015; Dimitrova-Shumkovska et al. 2020a), our recent findings of increased BP after carcinogen treatment suggest mitochondrial damage in hepatocytes, indicative of elevated inflammation and/or increase in the apoptosis rates. Results about the increased TSPO binding correspond

Table 2 TSPO Bmax (fmol/mg) and Kd value (nM) of control, DMBA- exposed, and DMBA-exposed + *P. torulosus*-administered mice

Vehicle control			DMBA exposed			DMBA exposed + *P. torulosus* administered		
Bmax (fmol/mg)	Kd (nM)	*n*	Bmax (fmol/mg)	Kd (nM)	*n*	Bmax (fmol/mg)	Kd (nM)	*n*
5835 ± 956	2.01 ± 0.58	7	11,838 ± 1771**	0.81 ± 0.07**	6	8135 ± 969$^{\#\#}$	0.88 ± 0.09	8

Data are presented as means ± SD. Data were analyzed with Mann–Whitney U test. Levels of Statistical significance: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus vehicle control; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ versus DMBA exposed group

with those published by Hatori et al. (2015) who detected greater ^{18}F-FEDAC uptake in the fibrotic septa and in activated macrophages of carbon tetrachloride (CCl_4)-treated rats. This indicates that TSPO elevated BP marks ROS overproduction and/or pro-inflammatory response in hepatocytes. Dimitrova-Shumkovska et al. (2010b) also reported significantly increased TSPO response in testicular and cardiac tissues after acute exposure of DMBA, thus confirming that TSPO in various tissues is prone to oscillation in its BP after chemically induced oxidative damage.

Oral treatment with water extract from *P. torulosus* resulted in significant decreases in Bmax ($p < 0.01$, Fig. 1e, f) of TSPO-specific ligand ^{3}H-PK11195, thus potentially reducing the hepatotoxic effect of DMBA. Binding affinity of ^{3}H-PK11195 was unaltered after treatment with fungal extract. Obtained values for the Bmax and Kd are also in corroboration with previously published results (Gavish et al. 1999; Thompson et al. 2013). Furthermore and in relation to the decrease in TSPO binding potential, one can assume inhibition of cardiolipin peroxidation, a decline in Ca^{2+} efflux into the cytosol, and prevention of apoptosis, thus preserving hepatic functionality (Fig. 2).

Studies About the Hepatoprotective Effects of *Phellinus* spp.

Hepatoprotective effects of members of *Phellinus* spp., particularly *P. igniarius* in a model with alcohol-induced liver failure, indicate reduced expression of inflammatory proteins involved in liver fibrosis, such as TNF-α, iNOS, and cyclooxygenase-2 (Choi et al. 2006). A recent study confirms that fungus *P. igniarius* stimulates the Farnesoid X receptor, a nuclear receptor involved in regulating the synthesis, absorption, and conjugation of bile acids, thus having a significant impact on cellular cholesterol and triacylglycerol (TAG) uptake, thereby counteracting hypercholesterolemia and restoring hepatic functionality (Dong et al. 2018).

Novel findings reveal that methanolic extracts from *P. pini has* inhibitory activity on pancreatic *cholesterol esterase*, which effectively delays and/or restricts intestinal cholesterol absorption. This suggests impact of another mechanism for hypocholesterolemic properties of the observed fungus confirmed by the significantly reduced levels of plasma cholesterol, TAG, and low-density lipoprotein (LDL) in

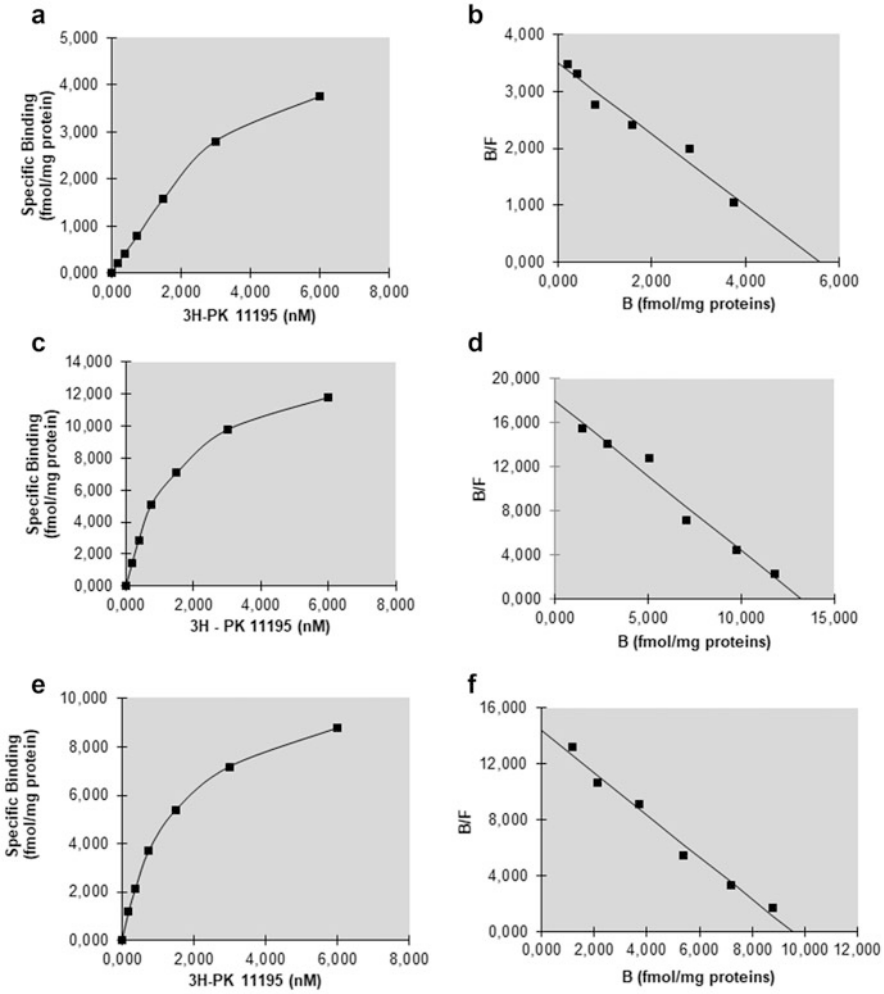

Fig. 1 Variations in TSPO BP after DMBA and *P. torulosus* administration. Variations in TSPO BP after DMBA and *P. torulosus* administration. Representative saturation curves (**a**, **c**, and **e**) and Scatchard plots (**b**, **d**, and **f**) of ^{3}H-PK11195 binding in liver membrane homogenates respectively of control mice (**a** and **b**), DMBA (**c** and **d**), and DMBA + *P. torulosus*-treated mice (**e** and **f**); *B* bound, *B/F* bound over free

high-fat diet-treated rats (Im et al. 2018). In accord, the treatment with *P. torulosus*, as indicated in our study, reverted the hypercholesterolemic effects of DMBA by significantly reducing plasma cholesterol and TAG levels (Table 3). These findings might provide the answer for the reduced TSPO density, measured in liver mitochondria in our study, additionally promoting *Phellinus spp*. as safe prophylactics in treating hyperlipidemia and obesity.

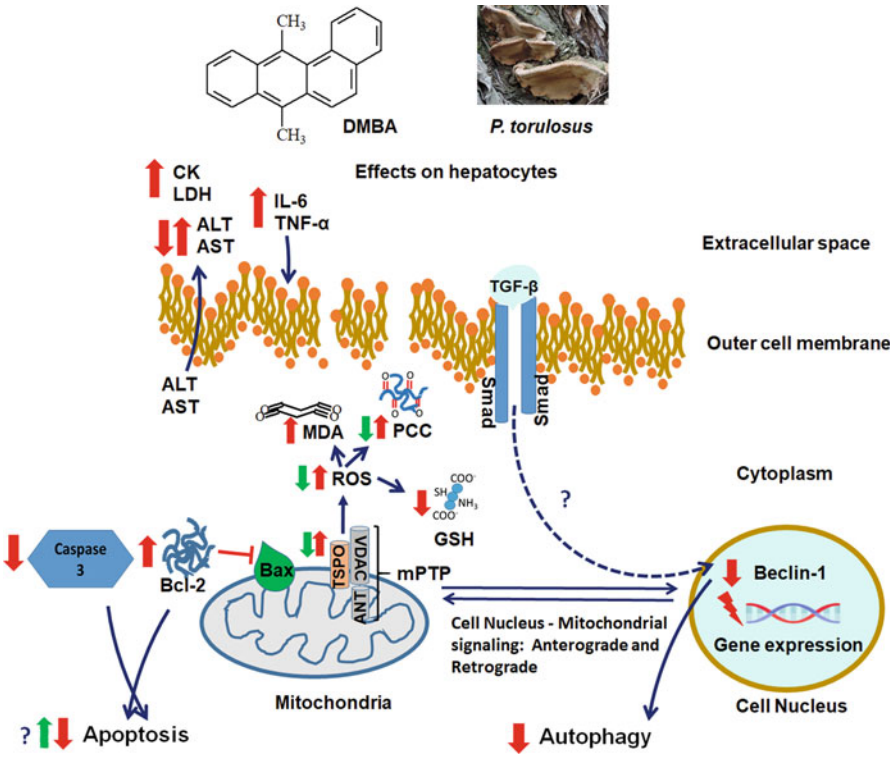

Fig. 2 DMBA hepatotoxic effects evidenced by fluctuations of transaminase levels, increase in pro-inflammatory and ROS markers, and inhibition of autophagy and apoptosis. Possible hepatotoxic and carcinogenic effects of environmental toxicant, DMBA evidenced by extracellular increase of transaminases, CK, LDH and inflammatory cytokines, intracellular increase in TSPO BP, ROS formation, and suppression of autophagy and apoptosis via reduced expression of caspase-3 and Beclin-1 with possible involvement of TGF-β – Smad signalling pathway and upregulation of Bcl-2 (red arrows) (Hosny et al. 2021). Treatment with *P. torulosus* significantly reduces TSPO BP, thus decreasing ROS which may lead to normalization of apoptosis rate (green arrows). Abbreviations: *ROS* reactive oxygen species, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *ANT* adenine nucleotide translocator, *CK* creatine kinase, *DMBA* 7,12-dimethylbenz[a] anthracene, *GSH* reduced glutathione, *LDH* lactate dehydrogenase, *IL-6* interleukin-6, *MDA* malondialdehyde, *ROS* reactive oxygen species, *PCC* protein carbonyl content, *TGF-β* transforming growth factor beta, *TNF-α* tumor necrosis factor alpha, *TSPO* 18 kDa translocator protein, *VDAC* voltage-dependent anion channel. (Modified from Dimitrova-Shumkovska et al. (2020b))

Applications to Prognosis, Other Diseases, or Conditions

Measurement of the inflammatory and oxidative damage biomarkers in clinical practice is useful in obtaining evidence about the adverse effects of environmental toxicants on human health, specifically effects on metabolic rate and cardiovascular and lung diseases. In this regard, the recent scientific focus is placed on

Table 3 Plasma lipid status of control, DMBA-exposed, and DMBA-exposed + *P. torulosus*-administered mice

Parameter	Vehicle control	DMBA exposed	DMBA exposed + *P. torulosus* administered
Total cholesterol (mg/dL)	76.88 ± 4.84	83.99 ± 3.86*	72.97 ± 4.60$^{\#\#}$
TAG (mg/dL)	76.85 ± 7.16	73.54 ± 2.24	60.49 ± 6.14$^{\#\#}$

Data are presented as means ± SD and analyzed with two-sided Student's t-test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ versus control group; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ versus DMBA exposed group

noninvasive methods to assess the oxidative damage through evaluation of urinary lipid peroxide products and DNA damage by 8-hydroxy-2′-deoxyguanosine (8-OHdG) in addition to PAH concentration in patients with chronic obstructive pulmonary disease (COPD), emphasizing the negative correlation between the oxidative damage and lung function (Liu et al. 2021). Moreover, findings about the association of elevated NO levels, iNOS, and MPO activity with various pathological conditions such as diabetes, insulin resistance, hypertension, and renal dysfunction stipulate their immense prognostic and diagnostic value for these disorders (Heinecke and Goldberg 2014; Bahadoran et al. 2019). It has been reported that the peroxynitrite generated from toxic amounts of NO possesses significant pro-oxidant activity, which is prone to oxidize LDLs, thus favoring the production of lysophosphatidylcholine. The latter molecule reduces eNOS activity by decreasing L-arginine uptake by endothelial cells, which in turn results in increased superoxide radical levels that regenerate peroxynitrite, this reveals a vicious circle that leads to the progression of acquired pneumonia to coronary heart disease (Ostrovskyy et al. 2020). Studies also reveal that MPO catalyzes the chlorination and nitration of HDL and oxidation of methionine residues in the apolipoproteins apoA1, C-II, and C-III associated with generation of pro-inflammatory HDL which contributes to the accumulation of TNF-α and IL-6, thus propagating the formation of atherosclerotic lesions (Witkowski et al. 2019). Moreover, increased levels of mentioned inflammatory markers are probably the cause for the reduction of lecithin-cholesterol acyltransferase (LCAT) activity which participates in cholesterol esterification and plays a crucial role in the process of HDL maturation (Márquez et al. 2020). Serum amyloid A (SAA), as well as other molecules synthesized in chronic inflammatory processes is capable of modifying HDL structure by displacing ApoA-1 and paraoxonase-1 (PON-1), resulting in its complete functional loss (Márquez et al. 2020).

As mentioned above, given the variety of regulatory functions of TSPO in processes crucial for "cellular life or death," recent publications target this mitochondrial receptor as a potentially useful tool in diagnosing neurodegenerative and psychiatric disorders, as well as traumatic brain injury (Dimitrova-Shumkovska et al. 2020a). In addition, it was also ascertained that many TSPO ligands, such as

PK11195, etifoxine, emapunil, and 2-Cl-MGV-1, demonstrate the potential of targeting TSPO for treatments of these diseases, thereby significantly increasing the scientific interest in discovering TSPO influences in PAH-derived oxidative stress, inflammation, and cell death given TSPO's ubiquitous expression, including distinct expression in the liver (Dimitrova-Shumkovska et al. 2020a). Thus, *P. torulosus* appears to be beneficial in ameliorating effects of provoked liver injury which may in part be due to its effects on TSPO expression.

Mini-dictionary of Terms

- **PAHs** – Persistent environmental toxicants produced from suboptimum combustion of organic materials predominantly found in $PM_{2.5}$ particles
- **DMBA –** PAH member with well-established hepatotoxic and carcinogenic effect in lung, breast, and skin tissues confirmed in animal models
- **Inflammation –** Defensive mechanism triggered by PAH that involves increase in pro-inflammatory cytokines, NO, MPO, and other molecules in exposed tissues
- **TSPO –** A valuable biomarker for inflammation and apoptosis localized in the outer mitochondrial membrane which regulates/alters diverse processes such as cholesterol transport, ROS formation, and cellular apoptosis
- ***Phellinus torulosus* –** Medicinal fungus known for its antimicrobial and antioxidant effects

Key Facts of PAH-Derived Inflammation

- The human population is exposed daily to PAHs as ubiquitously present pollutants through air, water, and food, thus enlarging the risk of cardiovascular or lung diseases which manifest themselves by the process of inflammation.
- Significantly toxic metabolites of PAH also induce liver damage and ROS buildup which is the main cause for activating pro-inflammatory pathways.
- Gaseous transmitter like NO and leukocyte-derived enzyme as MPO, recently added to the list of biomarkers, are providing new evidence about mechanisms of inflammation.
- NO levels are increased as a result of the activation of iNOS responding to tissue injury, and the produced RNS participate in activation of pro-apoptotic pathways, thus facilitating inflammation.
- MPO activity which favors H_2O_2 elimination is also stimulated during inflammation in order to inhibit the infiltration of mononuclear cells, thus generating numerous reactive pro-oxidants capable of causing severe damage to biomolecules.
- TSPO presents interesting mitochondrial biomarker connected with ROS formation and cellular integrity effective in diagnosing inflammation and response to therapy.

Summary Points

- PAH exposure causes liver damage by direct exposure via (i) ROS formation manifested by lipid peroxidation, protein modification, and DNA damage and (ii) inflammation by increasing levels of pro-inflammatory cytokines, NO, and MPO.
- AhR is also a significant contributor to the PAH-derived inflammation by activating expression of pro-inflammatory genes and NF-κB pathways.
- Blood, tissue, and mitochondrial biomarkers give a highly accurate image about the degree of exposure and should be regularly assayed in clinical practice.
- TSPO can potentially be utilized as a marker for hepatic diseases and be a diagnostic target to assay *P. torulosus*' beneficial effects on hepatic disorders.
- Acute dosages of medicinal fungi such as *Phellinus torulosus* can constrain oxidative damage and revive liver integrity after mild DMBA exposure.
- The significant reduction of TSPO BP caused by *Phellinus torulosus* administration is additional trigger for elucidation of protective actions of natural antioxidants on mitochondrial function.

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Oxidative Stress Biomarkers and Their Applications to Detect Excessive Fluorine

10

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Abstract

The review summarizes current data on the effects of fluoride ions on humans and mammals as well as current literature data on the molecular biomarkers of the physiological and toxic effects of fluorine compounds on the organism. It has been evidenced that fluoride ions can have a positive effect on the skeletal system and tooth enamel, the molecular mechanism of which is the formation of more acid-resistant fluorine-apatites. However, their positive effect is possible only in micromolar doses. At the same time, even at these concentrations, the generation of free radicals and, as a consequence, the activation of mitogen-activated protein kinase (MAPK)-dependent apoptosis pathways increase in all other organs and systems. In addition to apoptotic changes in various organs and tissues, the processes of lipid peroxidation are intensified. The functioning of the nitric oxide cycle is shifted towards the activation of inducible forms of NO synthases, reducing the arginase pathway of L-arginine metabolism. Fluoride can cause the simultaneous development of several types of hypoxia. The analysis of numerous studies revealed that fluorine compounds are a cytotoxic factor involved in changes in metabolism, modulation of intracellular signaling pathways, and activation of programmed cell death. In this case, the mechanisms of the physiological or toxic effects of fluorine compounds on the organism depend on their concentration and duration of exposure. The development of some of the most threatening effects of fluorides is dose-dependent and requires millimolar concentrations. Given the data from the last decade, a question arises of the effectiveness of water fluoridation as a means of prevention of dental diseases. Therefore, analyses of the molecular mechanisms of fluoride exposure, despite the research conducted so far, are promising tools to elucidate the general mechanisms of fluoride-associated diseases.

Keywords

Fluoride · Toxic effects · Cell redox status · Cell membrane permeability · Transcription · Translation · Lipid peroxidation · Antioxidant defenses · Intracellular signaling pathways · Apoptosis

Abbreviations

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
Bcl-2	B cell lymphoma 2
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
CD	Cluster of differentiation
cGMP	Guanosine monophosphate
CHOP	C/EBP (enhancer-binding proteins) homologous protein
CytoC	Cytochrome c
DAG	Diacylglycerol
GDP	Guanosine diphosphate

GPR78	G-protein-coupled receptor 78
GPx	Glutathione peroxidase
GR	Glutathione reductase
H_2O_2	Hydrogen peroxide
HDL	High-density lipoproteins
HF	Hydrogen fluoride
HIF-1	Hypoxia-inducible factor 1
HPA	Hypothalamic-pituitary-adrenal system
Hsp70	Heat-shock proteins 70
IL	Interleukin
IQ	Intelligence quotient
IRE1	Inositol-requiring enzyme 1
IκB	Inhibitor of nuclear factor kappa B
LPO	Lipid peroxidation
MAPK	Mitogen-activated protein kinase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NOS	NO synthase
O_2	Superoxide anion radical
PTH	Parathyroid hormone
ROS	Reactive oxygen species
SOD	Superoxide dismutase
T3	Triiodothyronine
T4	Thyroxine
TBARS	2-Thiobarbituric acid-reactive substances
TSH	Thyroid-stimulating hormone
α-GPDH	α-Glyceraldehyde phosphate dehydrogenase

Introduction

Fluoride is one of the most common elements on Earth. In a free state, fluorine does not exist in nature but forms inorganic and organic complex compounds – fluorides, the content of which in the Earth's crust is approximately 0.06–0.09% (Kirk 1991). Fluorine compounds are widespread in nature and are industrial pollutants. Fluorine can also be found in 296 different species of minerals, the most abundant of which are fluorspar, fluorapatite, topaz, and cryolite. The largest deposits of fluoro-minerals are found in China, Mexico, Mongolia, South Africa, and Russia, where populations inhabiting these areas suffer from endemic fluoride toxicity (Johnston and Strobel 2020).

It is known that low concentrations of fluoride are necessary for the normal growth and development of the body. Fluorine has a regulatory effect not only on bone tissue cells (osteoblasts and osteoclasts) but also on endothelial, liver, kidney, myocardial, and nervous system cells (Dhar and Bhatnagar 2009; Barbier et al. 2010). The daily requirement for fluoride is 1.5–4 mg. Toxic doses of fluoride for humans vary in a wide range: 16–64 mg/kg for adults and 3–16 mg/kg for children

(Barbier et al. 2010). The toxicity of fluoride is associated with its high chemical and biological activity.

The transport medium for fluorides in the organism is blood. The concentration of fluorides in plasma is 0.5–10.5 μmol/L, and approximately 72% of ionized fluorides are contained in blood plasma. The level of fluoride in blood plasma is mainly regulated by two systems, namely, the urinary excretory and skeletal systems. Fluoride reabsorption in the renal tubules is limited, and it is rapidly excreted in the urine. However, the main mechanism for the removal of fluoride from the bloodstream is a fixation by the skeletal system. Fluorine is actively involved in phosphorus-calcium metabolism. With a moderate intake of fluoride, the bones of adults contain 1–5 g/kg, and cancellous bones are richer in the element than tubular ones (Shalina and Vasilyeva 2009).

The bone plays an important role in regulating the concentration of fluoride in the extracellular fluid due to its ability to quickly bind excess fluoride and mobilize it into the extracellular fluid in the case of deficiency. As the pH decreases, the fluorine concentration declines. Fluorine combining with hydroxyapatite forms fluorapatite, although it accounts for only 1/40 of apatite. Nevertheless, it is precisely its presence that imparts acid resistance and strength to the teeth and bones. Fluorides promote calcium fixation in skeletal tissues and their mineralization; inhibit lipase, esterase, and lactate dehydrogenase; activate adenylyl cyclases; stimulate hematopoiesis; disrupt the fermentation of carbohydrates in the oral cavity; and destroy cariogenic bacteria (Shalina and Vasilyeva 2009).

Fluorine and its compounds are capable of accumulating in various objects in the environment in different quantities. In surface and underground sources of drinking water, the natural concentration of fluorine ranges from 0.05 mg/L to 15 mg/L, which is related to climatic, geographic, hydrogeological, and other conditions (Shalina and Vasilyeva 2009). The optimal fluoride content in drinking water is 0.7–1.1 mg/L, which is the World Health Organization limit followed in most of the nations (WHO 1985). There are regions where its content is much lower. For example, in the waters of England and Wales, the fluorine content does not exceed 0.3 mg/L (Fuge and Andrews 1988). In Central Europe, groundwater resources that exceed the upper guideline value of 1.5 mg/L are widespread in Ukraine, Moldova, and Hungary (Fordyce et al. 2007). Endemic fluoride toxicity in humans occurs from the chronic consumption of over 1.5 ppm (75 μM) fluoride (Johnston and Strobel 2020).

Sources of Fluoride Intake and Distribution in the Body

Fluoride ions can enter the human body with drinking water, especially in regions where the content of these ions in groundwater significantly exceeds the permissible values (Spencer and Do 2016). Foods, especially those of plant origin, can also be a source of excess fluoride ions (Jha et al. 2011). In recent years, fluoride has become increasingly relevant as a halogen drug modifier (Wu and Wan 2008). Since the van der Waals radius of the fluorine atom is between the hydrogen atom and the oxygen atom, the inclusion of this halogen in the structure of drugs increases their solubility in lipids and acid resistance, with an insignificant effect on their conformation

(Cavallo et al. 2016). Therefore, the iatrogenic fluoride load requires careful monitoring. Fluoride ions, having a high electronegativity, are able to form hydrofluoric acid (HF) in conditions of gastric acidity, which is much easier to pass through biological membranes (Buzalaf and Whitford 2011).

All foods contain fluoride. Some of the highest concentrated food sources are fluoride-accumulating plants, such as tomato, spinach, grapes, tea, and elderberry (Johnston and Strobel 2020). The daily intake of fluoride from food is on average up to 2–3 mg, 90–97% of which is absorbed through the gastrointestinal tract into the blood. From blood plasma, fluorine is rapidly distributed in intracellular and extracellular fluids, tissues, and organs. Fluorine is able to rapidly penetrate biological membranes in the form of hydrogen fluoride (HF) through passive diffusion (Buzalaf and Whitford 2011). Experiments with radioactive fluorine have shown that its intracellular concentration depends on the pH gradient and is 10–50% lower than in blood plasma. Equilibrium fluorine concentrations are reached faster between plasma and well-supplied organs (heart, lungs, and liver) than between plasma and skeletal muscles, skin, and other organs. As a result, fluoride compounds are distributed in the body as follows: bone tissue> tooth enamel> dentin> parenchymal organs. Fluorine as a chemical element does not undergo metabolic transformations but can either accumulate or only be excreted from the organism. Moreover, its maximum accumulation is shown in the kidneys; less fluoride is accumulated in the brain, and the element is absent in the liver. The elimination of fluoride from the body occurs through the skin, digestive tract, and urinary system. The half-life of fluoride elimination from the body is 2–9 hours (Buzalaf and Whitford 2011).

There are different forms of inorganic fluoride in animal and human tissues:

(i) Fluorine in the form of free ions, which can be measured using ion-selective electrodes
(ii) Fluorine in the form of compounds and complexes, including organometallic ones, such as (a) HF at low pH values; (b) complexes of fluorine bound to metal ions (Ca^{2+}, Mg^{2+}, Fe^{3+}, Al^{3+}, etc.); (c) fluorine absorbed in mineral-organic sediments, for example, in saliva; and (d) fluorine incorporated into the apatite elements of the bones and teeth.

All of these inorganic fluorine fractions can be converted to ionic inorganic fluorine, and their content can be measured (Venkateswarlu 1994; Shalina and Vasilyeva 2009).

The selectivity of fluorine compounds to damage highly mineralized tissues, in particular the bones of the skeleton and teeth, is well known (Wei et al. 2019). However, fluorosis refers to polysystemic diseases in which pathological changes are observed in many organs affecting the liver, kidneys, and neuroendocrine, cardiovascular, and skeletal systems (Zhou et al. 2015) (Fig. 1).

Currently, the question of the biogenic effect of fluorine at the cellular level remains open, since the required amount is close to the dose causing the damaging effect. It has been shown that the effects of fluoride on the physiological functions of the body and cellular metabolism depend on the type of cells, concentration, and duration of action (Barbier et al. 2010). For example, in bone and dental tissues,

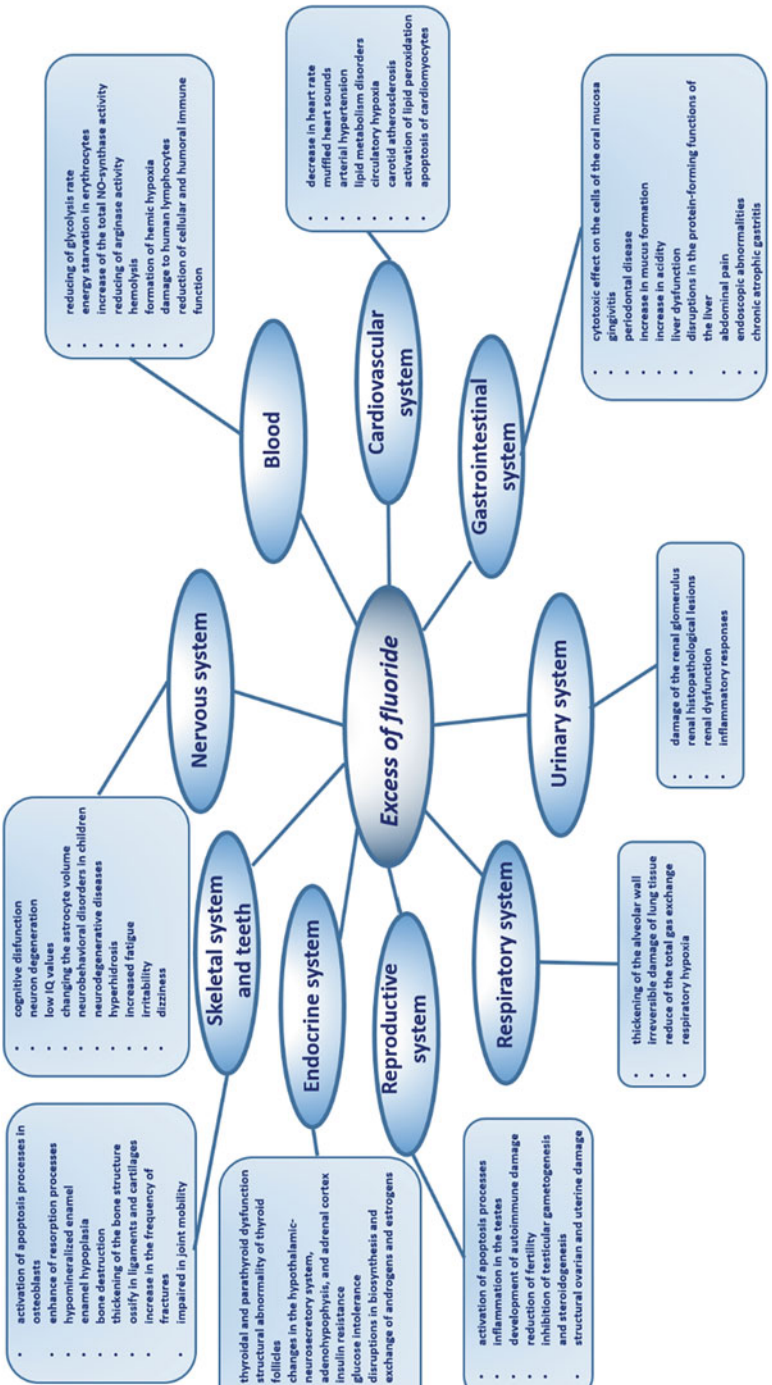

Fig. 1 Adverse health effects on human beings due to excess fluoride intake

fluorine in micromolar concentrations causes cell proliferation and growth, while its millimolar concentrations inhibit proliferation and induce cell apoptosis (Oka et al. 2020). Controlled exposure to fluoride increases overall teeth quality through enamel replacement and the killing of plaque-causing bacteria (Johnston and Strobel 2020).

Influence of Fluorides on Organs and Systems

Effects on the Skeletal System and Teeth

The positive effect of fluoride ions on the formation of enamel has been well studied. Fluorine, as a more electronegative ion, has the ability to displace the hydroxyl group from the hydroxyapatite molecule, thus forming a new type of apatite – fluoride. This type of apatite is more resistant to acids formed in the oral cavity and has greater microhardness (Arnold et al. 2007; Everett 2011). Fluorides can affect the rate of remineralization of the teeth, especially in conditions of fluorosis. Alhawij and co-authors (2015) showed that the teeth affected by fluorosis have a higher ability to remineralize than intact. However, this effect is observed only in the presence of excess fluoride ions (Alhawij et al. 2015). Also, the acidity in the oral cavity affects the rate of the substitution of the hydroxyl group to the fluorine ion. Therefore, the optimal pH value (4.5–5.1) increases the rate of substitution, and calcium fluoride can also be formed during the reaction (Arnold et al. 2007). Since hydroxyapatites are also present in bone tissue, a similar process is possible in the skeleton and is likely to increase bone strength by increasing their microhardness (Everett 2011).

Nevertheless, it is important to note that the processes of mineralization and demineralization of the bones and teeth follow different mechanisms (Abou Neel et al. 2016). Tooth enamel in the formed tooth does not contain cells capable of performing remineralizing functions; hence, this process depends on the mineral composition of the oral fluid. There are also data on the remineralizing effect of dental cerebrospinal fluid exiting the pulp through the dentin to the enamel. The bones are composed of cells that control the processes of remineralization and demineralization. Osteoblasts are responsible for mineralization, while osteoclasts resorb bone. Sodium fluoride at micromolar concentrations is able to inhibit the differentiation of progenitor cells into osteoclasts in conditions of periodontitis. This effect is possible by inhibiting matrix metalloproteinases (MM9 in particular) and by reducing the activation of the receptor activator of the NF-κB ligand (22 RANKL). Thus, fluoride ions help reduce bone resorption. On the contrary, increasing the concentration of fluoride ions to millimolar values (1 mM and above) enhances resorption processes and can lead to bone destruction. The mechanism of this phenomenon is associated with the activation of apoptosis processes in osteoblasts (Everett 2011; Zhang et al. 2015). This effect is associated with an increase in membrane potential and an increase in mitochondrial membrane permeability. This creates the conditions for the entry of cytochrome c into the cytoplasm. Fluoride ions have also been shown to enhance BCL-2 expression and activate caspases 8, 9, and

10 (Zhang et al. 2015). At a concentration of 50 μM, fluoride ions are able to increase the production of superoxide anion radicals in the mitochondria of osteoblasts while reducing their use of oxygen. Increased production of reactive oxygen species (ROS) occurs in the conditions of reduced activity of antioxidant defenses, i.e., superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), dramatically reducing their activity under fluoride intoxication (Gutiérrez-Salinas et al. 2013). Chondrocytes also suffer from toxic damage, which creates conditions for cartilage resorption (Meng et al. 2014). Thus, the effect of fluoride ions on the skeletal system depends on the dose and can be both positive and negative.

Nervous System

The skeletal system is not the only system in the body that is affected by fluoride ions. There have been no data on their positive effect on the nervous system in the literature for the last 25 years, whereas their negative effect has been described in some detail. Thus, it is known that when the concentration of fluoride ions in drinking water exceeds 1.2 mg/L, the intelligence (according to the IQ test) of people is significantly reduced (Choi et al. 2012). The underlying mechanism of neurotoxicity consists in changing the astrocyte volume. This is caused by the activation of Cl- channels; excessive removal of chlorine from the cell alters the osmotic balance and leads to dehydration of the cell (Lee et al. 2016). Since the cell volume and ion channel function play an important role in nerve impulse transmission, the function of neurons in fluoride intoxication is significantly impaired. There is evidence that activation of these channels occurs with the participation of mitogen-activated protein kinase (MAPK). Fluoride ions are activators of G-proteins and phosphatase inhibitors; therefore, they are able to activate MAPK-dependent chlorine ion channels (Lee et al. 2016) (Fig. 1).

In subjects in contact with fluoride compounds, vegetative-vascular manifestations, i.e., hyperhidrosis, increased fatigue, irritability, and dizziness, are noted (Guo et al. 2008). It was found that drinking water with a high fluoride content throughout pregnancy negatively affects the neurobehavioral development of children (Li et al. 2008). The literature discusses data on the relationship of fluorosis with Alzheimer's disease and the high content of Al and F in brick tea, which is prepared in China from low-quality tea, mainly from old tea leaves. Aluminum and fluorine enter the tea leaf from acidic soils (Wong et al. 2003).

Blood and Cardiovascular System

Fluoride ions are able to reduce the rate of glycolysis in whole blood. This effect can be explained by the inactivation of enolase. Since erythrocytes are unable to use the aerobic glucose oxidation pathway, inactivation of the anaerobic pathway will lead to energy starvation in erythrocytes, which in turn will damage them by hemolysis due to disruption of ATP-dependent ion channels (Shashi and Meenakshi 2015).

Increased hemolysis of erythrocytes creates conditions for the formation of hemic hypoxia. There is evidence of damage to human lymphocytes by fluoride ions at micromolar concentrations (<1 μM). The mechanism of damage, in this case, is to increase the generation of free radicals. This creates conditions for changing the functioning of the immune system towards the reduction of immune resistance (Jothiramajayam et al. 2014).

It was experimentally established that fluoride ions are able to affect the functioning of NO synthases (NOS) and arginases in the blood. Fluoride ions are able to increase the total activity of NOS while reducing the activity of arginases. Excessive production of nitric oxide (NO) can increase fluoride-induced oxidative stress due to the formation of peroxynitrite. The arginase pathway of L-arginine metabolism leads to the formation of polyamines (putrescine, spermidine, spermine), which are involved in mitotic cell division. The inhibition of this pathway will reduce the regenerative properties of tissues (Akimov et al. 2015). The nitrite content increases in these conditions. Nitrites are oxidized derivatives of NO, which are formed due to the enhanced activity of NOS. Nitrites can bind to blood plasma proteins, reducing their functional activity or completely inactivating these proteins (Akimov et al. 2015).

Given the changes in the use of oxygen by mitochondria in fluoride intoxication, tissues that require large amounts of oxygen to function suffer significant damage. Muscle tissue in general and heart muscle, in particular, are significantly damaged by fluoride intoxication (Panneerselvam et al. 2017). The processes of necrosis due to increased lipid peroxidation and apoptosis in muscle tissue occur simultaneously. Damage to the heart muscle creates conditions for the gradual development of circulatory hypoxia (Fig. 1).

Long-term exposure to fluorine compounds is one of the risk factors for the development of arterial hypertension and lipid metabolism disorders and an increase in the content of cholesterol and high-density lipoproteins (HDL) in the blood serum (Umarani et al. 2015). There is a decrease in heart rate and muffled heart sounds in patients with fluorosis (Karademir et al. 2011).

Gastrointestinal System

With the oral intake of fluorides, functional changes in the organs of the digestive tract are noted. Dental fluorosis occurs in children and adults with prolonged use of water, tea, or milk containing fluorides at a level of 1.5 mg/L and above (Srivastava and Flora 2020). Sodium fluoride has a cytotoxic effect on the cells of the oral mucosa, causing gingivitis, periodontal disease, and extremely rarely ulcerative-necrotizing gingivitis. It has been experimentally proven that aging cells of the oral mucosa, in comparison with young cells, become resistant to the cytotoxic effect of sodium fluoride in vitro. In rats, an increase in the secretory activity of the gastric mucosa was observed, which was manifested by an increase in mucus formation and an increase in acidity. Excessive fluoride intake disrupts the protein-forming functions of the liver (Chattopadhyay et al. 2011). In the blood serum, the content of

β-globulins and albumin, haptoglobin, and C-reactive protein are increased. All patients with osteofluorosis analyzed in the study conducted by Dasarathy and co-workers (1996) had gastrointestinal symptoms, the most common being abdominal pain, endoscopic abnormalities, and chronic atrophic gastritis (Fig. 1). These included loss of microvilli, cracked-clay appearance, and the presence of surface abrasions on mucosal cells (Dasarathy et al. 1996). Also, fluoride ions are able to reduce important biochemical parameters of the salivary glands. Picco and co-authors (2014) showed that the addition of 20 ppm NaF to drinking water for 30 days increases salivation. The concentration of calcium ions in the secretion of salivary glands also decreases (Picco et al. 2014). Fluoride has hepatotoxic effects. It can cause liver dysfunction, interfere with the balance of antioxidant activity, and induce morphological changes in the liver (Zhou et al. 2015). Also, chronic exposure to fluoride causes damage to liver histopathology and leads to liver apoptosis through caspase-mediated pathways promoting the relative expression of caspase-3 and caspase-9 proteins and DNA damage. Fluoride also can cause oxidative stress in the liver (Wei et al. 2014).

Urinary System

The kidneys suffer from the increased generation of superoxide anion radicals and the degradation of antioxidant defenses. In the kidneys, the fluoride-mediated activation of apoptosis is less pronounced. The use of antioxidants significantly reduces damage caused by fluoride ions (Yadav et al. 2016). Renal pathological studies have shown varying degrees of fluoride-associated damage to the architecture of tubular epithelia, endothelial cells, and mesangial cells of the renal glomerulus. Fluoride-induced oxidative stress and the NF-κB signaling pathway play an important role in the development of renal damage, which may eventually result in renal histopathological lesions and inflammatory responses (Zhang et al. 2014) (Fig. 1).

Respiratory System

There is also evidence of irreversible damage to lung tissue, manifested by thickening of the alveolar wall on the one hand (Fig. 1). The thickened alveolar wall contains an increased number of macrophages. On the other hand, part of the alveolar septa collapses, increasing the amount of air space. This reduces the total gas exchange surface, which can lead to respiratory hypoxia (Abdel-Gawad et al. 2014).

Reproductive System

Because the increased generation of free radicals with reduced activity of antioxidant systems leads to the activation of lipid peroxidation (LPO), which is a universal

mechanism of cell damage, there are conditions in which cell contents will enter surrounding tissues, stimulating inflammatory response. The mechanisms of apoptosis, which are also triggered by fluoride intoxication, play a protective role in this case, as they reduce the inflammatory response. On other hand, the activation of apoptosis processes in tissues that are constantly in the process of division is difficult. This may explain the development of inflammation in the testes under fluoride intoxication, as spermatogenesis occurs constantly throughout the life of mammals (Wei et al. 2016). Since mammalian testicular tissue is located behind a specific barrier, its immunological tolerance does not develop; hence, its inflammation may result in the development of autoimmune damage. This underlies the reduction of fertility in conditions of chronic fluoride intoxication (Fig. 1).

With a chronic intake of an increased amount of fluorides, the biosynthesis and exchange of androgens and estrogens are disrupted, which is expressed in a decrease in testosterone production accompanied by increased generation of estrogens and violation of the endocrine function of gonads. Fluoride exposure was found to lead to a decrease in male reproductive potentials in rats and rabbits, as well as inhibition of testicular gametogenesis and steroidogenesis in association with oxidative stress in the testis and male accessory sex organs in rats. Female reproductive function is inhibited by NaF, and exposure to NaF causes structural ovarian and uterine damage. NaF may thus significantly reduce the fertility of female rats (Zhou et al. 2013). A negative correlation was found between the level of apoptosis in spermatogenic cells and the level of testosterone and estradiol. High concentrations of fluorides entering the body of white rats with water led to a decrease in the relative mass of their testes; a decrease in the number, motility, and survival of spermatozoa; and an increase in the number of abnormal spermatozoa (Pushpalatha et al. 2005).

Endocrine System

Subacute exposure to sodium fluoride at a dose of 20 mg/kg b.w./day orally to rats for 30 days was found to induce thyroidal dysfunction, including suppressed synthetic machinery of the thyroid gland to produce nucleic acids and thyroid hormones, mainly T3 and T4. The structural abnormality of thyroid follicles caused by fluoride intoxication clearly indicates its thyrotoxic effect. There are morphological studies indicating consistent changes in the hypothalamic-neurosecretory system, adenohypophysis, and adrenal cortex during fluoride intoxication (Fig. 1). A decrease in the morphofunctional activity of the adrenal cortex and adenohypophysis has been shown, which may be manifested by inhibition of the secretion of hormones of the hypothalamic-pituitary-adrenal system (HPA). The result of such hormonal changes is the development of metabolic insufficiency in tissues (Agalakova and Gusev 2011).

It has been shown that fluoride intoxication increases the functional activity of the main cells of parathyroid glands and C cells of the thyroid gland. As a result, the level of hormones regulating Ca^{2+} homeostasis in the body, i.e., parathyroid hormone (PTH) and calcitonin, is increased (Fig. 1). Excessive secretion of PTH in response to fluoride intoxication was reported, i.e., the level of this hormone in the blood of experimental rats exceeded the control values by five times. It has now been

shown that high levels of PTH increasing the Ca^{2+} current into cells contribute to uncoupling of oxidative phosphorylation, reduce the formation of ATP, and have an adverse effect on lipid and carbohydrate metabolism (Koroglu et al. 2011).

Long-term action of fluorine leads to a change in the content of thyroid-stimulating hormone (TSH) and thyroid hormones – triiodothyronine (T3) and thyroxine (T4) – in the blood. Moreover, the dynamics of the level of these hormones depend on the duration of the action of the damaging factor. The first phase is characterized by an increase in the level of both TSH and T4 in the blood accompanied by a reduced content of T3. Then, the level of TSH and thyroid hormones returns to the control values, and, along with the duration of the action of the damaging factor, the phase of inhibition characterized by a decrease in the level of TSH, T4, and T3 begins (Wang et al. 2009). Thyroid hormones regulate redox processes and basal metabolism, as a result of which they provide a more intensive functioning of the whole organism under the influence of a damaging factor. Thus, with fluoride intoxication, there are multidirectional changes in the content of important adaptive hormones in the blood, i.e., TSH, T3, T4, PTH, calcitonin, etc. In this case, the phase changes in the content of these hormones reflect the compensatory response of the organism in response to the prolonged action of the fluorine compounds. The serum fluoride concentration also has a significant relationship with thyroid hormone (FT3/FT4) and TSH concentrations. A positive correlation exists between fluorosis and thyroid functional activity (Kumar et al. 2018).

A significant increase in the serum insulin level and a general decrease in the glucagon level were reported, and histomorphometry analysis indicated an elevated insulin-positive area and an increase in the islet size in rats treated with fluoride for 1 year. In addition, fluoride obviously facilitated the mRNA expression of insulin receptors in vitro (Hu et al. 2012).

Immune System

NaF can reduce blood cellular and humoral immune function in mice. Alterations in blood cellular immunity and humoral immunity in mice were studied by Guo and co-workers (2017). In the case of cellular immunity, these authors found that sodium fluoride applied in excess of 12 mg/Kg resulted in a significant decrease in the percentages of CD3+, CD3 + CD4+, CD3 + CD8+ T lymphocytes in peripheral blood. Additionally, serum T helper type 1 (Th1) cytokines including interleukin (IL)-2, interferon (IFN)-γ, tumor necrosis factor (TNF), and Th2 cytokines including IL-4, IL-6, IL-10, and Th17 cytokine (IL-17A) contents were decreased. In the case of humoral immunity, NaF reduced the peripheral blood percentages of CD19+ B lymphocytes, serum immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM) (Guo et al. 2017).

Skin

The epithelium does not avoid damage caused by fluoride intoxication. Changes in the epithelium are associated with activation of lipid peroxidation and apoptosis.

Significant DNA damage was found in epithelial cells. Morphologically, such damage is manifested as erosions and ulcers (Susik et al. 2015).

Oxidative Stress Biomarkers and Applications to Detect Excessive Fluorine

Biomarkers of Cell Redox Status

Maintaining redox homeostasis is important for the vital activity of cells since its violation is accompanied by an increase in the level of ROS leading to damage to lipids, DNA, and proteins. Fluorine is a prooxidant – under its action, the generation of $\cdot O_2^-$, H_2O_2, OH·, and nitric oxide (NO) increases in cells (García-Montalvo et al. 2009; Hassan and Yousef 2009). Oxidative stress is one of the mechanisms of fluorine cytotoxicity, which has been shown in various experimental models. Fluorine compounds inhibit the activity of antioxidant enzymes – superoxide dismutase, catalase, and glutathione peroxidase. Disruption of the balance of pro- and antioxidants leads to activation of free radical processes and damage to membrane structures of cells in various organs and tissues (Bharti et al. 2014; Hassan and Yousef 2009) (Fig. 2).

Long-term high fluoride intake at the early stages of life enhances oxidative stress in the blood, thereby disturbing the antioxidant defense, as evidenced by a high level of glutathione transferase and a low glutathione level in the blood of rats subjected to chronic fluoride toxicity (Shivarajashankara et al. 2003). High concentrations of fluorine lead to intoxication of the organism, as evidenced by a high level of glutathione transferase and low glutathione reductase in the saliva of patients with fluorosis (Gavriliuk et al. 2007). Children exposed to fluoride (>1.5 ppm) through drinking water for more than 5 years had higher blood levels of 2-thiobarbituric acid reactive substances (TBARS), while acyl hydroperoxide levels were non-significantly increased in comparison with healthy children living in a non-fluorosis area (Tkachenko et al. 2021). A decrease in the activity of glutathione reductase during intoxication with fluorine may indicate suppression of the pentose phosphate pathway of glucose oxidation. Thus, in patients with fluorosis, the activity of the key enzyme of this pathway of glucose metabolism, i.e., α-glyceraldehyde phosphate dehydrogenase (α-GPDH), was reduced by 35% (Gavriliuk et al. 2007).

The glutathione reductase (GR) and α-GPDH enzymes are conjugated, since approximately 65% of $NADPH_2$ formed in the pentose phosphate pathway of glucose oxidation is used by glutathione reductase and for detoxification of toxins (monooxygenase pathway), lipid synthesis, steroid hormones, etc. Therefore, a decrease in the activity of the pentose phosphate pathway may reflect the suppression of anabolic processes during fluoride intoxication. In addition, there is an inverse relationship between the content of glutathione, the activity of glutathione transferase, and the degree of development of fluorosis. It was shown that the degree of damage caused by fluorosis correlated with a decrease in the content of glutathione and an increase in the activity of glutathione transferase in the saliva of patients (Gavriliuk et al. 2007). Thus, as a result of chronic fluoride intoxication, an

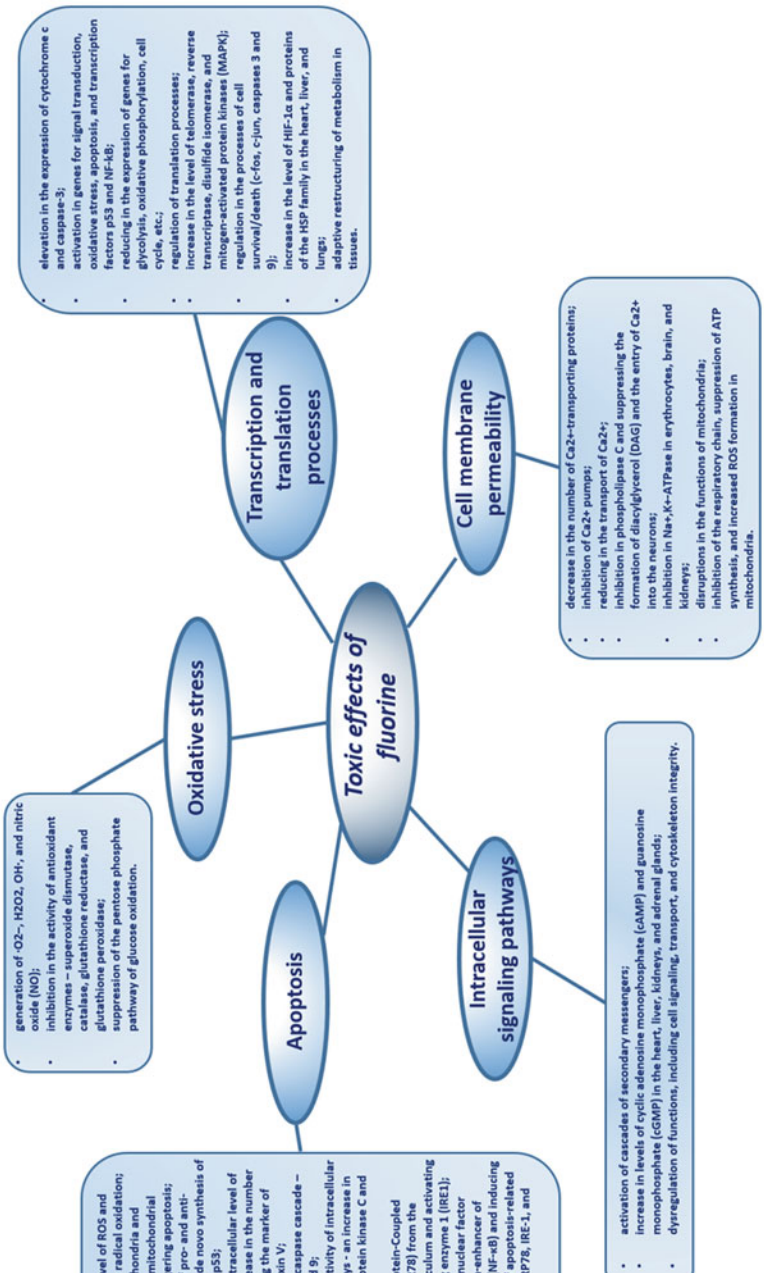

Fig. 2 Molecular mechanisms of fluoride toxicity

imbalance in the pro- and antioxidant systems is observed. At the same time, there is a correlation between clinical signs reflecting the degree of pathology and the activity of antioxidant defense enzymes (Fig. 2).

Transcription and Translation Processes

Among the most important mechanisms of action of inorganic fluorine compounds on the cell, the influence on the processes of transcription and translation is distinguished. Until recently, it has been believed that fluorine inhibits these processes. However, modern studies have shown the activation of transcription and translation in various tissues under fluoride exposure. It has been shown that fluorine compounds are transcription modulators in different types of cells (Salgado-Bustamante et al. 2010). Using the method of recombinant DNA, 183 genes were identified, the expression of which is altered by fluorine compounds. Fluoride exposure also significantly elevated the expression of cytochrome c and active caspase-3 proteins. In global gene expression profiling, 34 upregulated and 63 downregulated genes, which are involved in several sperm biological processes, including signal transduction, oxidative stress, apoptosis, electron transport, glycolysis, chemotaxis, spermatogenesis, and sperm capacitation, were significantly differentially expressed (Sun et al. 2011). The genes for signal transduction, oxidative stress, apoptosis, and transcription factors p53 and NF-kB are activated, while the expression of genes for glycolysis, oxidative phosphorylation, cell cycle, etc. is reduced (Fig. 2).

Inorganic fluorine compounds are also involved in the regulation of translation – de novo synthesis of 21 proteins in the heart, 28 in osteoblasts, and 13 in the kidneys was found (Lu et al. 2009). Most of these proteins in fluorosis regulate the oxidative metabolism of cells and the mechanisms of apoptosis. An increase in the level of telomerase, reverse transcriptase, disulfide isomerase (involved in protein folding), and mitogen-activated protein kinases (MAPK) has been shown (Lu et al. 2009). In addition, an increase in the level of protein factors that regulate the processes of cell survival/death, i.e., c-fos, c-jun, and caspases 3 and 9, has been reported (Zhan et al. 2006).

An organ-specific response to fluorine intoxication has been shown to be mediated by hypoxia-inducible factor 1 (HIF-1) and stress-inducible proteins of the HSP (heat-shock proteins) family. High concentrations of fluorine reduce the level of the transcription factor HIF-1α, which leads to suppression of the synthesis of protective proteins in liver cells, HSP70, and antioxidant defense enzymes such as SOD, catalase, and glutathione peroxidase in osteoblasts (Basha and Sujitha 2011). At the same time, the level of heat-shock proteins 70 (Hsp70) in the kidneys was found to be increased and correlated with the degree of damage to cells (Chattopadhyay et al. 2011).

On the contrary, under the subchronic action of fluorine on the body, an increase in the level of HIF-1α and proteins of the HSP family in the heart, liver, and lungs has been shown (Zhukova et al. 2011; Mikhaĭlova et al. 2012). An increase in the level of these protective proteins in the early stages of fluoride exposure was

accompanied by an adaptive restructuring of metabolism in tissues. The activity of enzymes ensuring the work of the Krebs cycle (aspartate aminotransferase) and lipid (hydroxybutyrate dehydrogenase) and protein (γ-glutamyl transferase) metabolism increased in the heart and lungs, and the glucose-alanine cycle enzyme (alanine aminotransferase) was activated in the liver (Zhukova et al. 2011; Mikhaĭlova et al. 2012) (Fig. 2).

Thus, in response to fluoride intoxication, the synthesis of various proteins is activated or suppressed in the cell, the qualitative composition of which depends on the concentration and duration of exposure to fluorine. Urgent response systems are activated, but the transcription and synthesis of structural proteins and enzymes that regulate metabolism are inhibited.

Biomarkers of Cell Membrane Permeability

It has been shown that fluorine ions change Ca^{2+} homeostasis in cells. Fluoride exposure reduces the transport of Ca^{2+} along the endoplasmic reticulum and through plasma membranes in kidney cells and in the sarcoplasmic reticulum of cardiomyocytes as a result of a decrease in the number of Ca^{2+}-transporting proteins and inhibition of Ca^{2+} pumps (Ono and Arita 1999). In the cells of the nervous system, fluorine inhibits the phospholipase C enzyme, suppressing the formation of a secondary messenger of diacylglycerol (DAG) and the entry of Ca^{2+} into the cell. However, in the cytosol of cells of other tissues (erythrocytes, osteoblasts, proximal tubules, fibroblasts, endothelial cells), fluoride intoxication has been shown to increase the concentration of Ca^{2+} (Agalakova and Gusev 2011) (Fig. 2).

Fluorine compounds inhibit Na^{+}, K^{+}-ATPase in the erythrocytes, brain, and kidneys or act as cotransporters, as a result of which phosphorus intake into the cell may decrease (Waugh 2019). Fluorine compounds disrupt the functions of mitochondria, causing a drop in membrane potential and the formation of a giant pore in their outer membrane (Aulestia et al. 2020). The consequence of the opening of the pore is the swelling of the mitochondrial matrix, rupture of the outer membrane of mitochondria, and release of cytochrome c from the intermembrane space. Loss of cytochrome c by mitochondria leads to inhibition of the respiratory chain, suppression of ATP synthesis, and increased ROS formation. In addition, the violation of the barrier properties of mitochondrial membranes under the influence of fluorine ions leads to apoptosis (Suzuki et al. 2015).

Biomarkers of Intracellular Signaling Pathways

One of the important systems through which the cell response to the action of environmental factors in normal and pathological conditions is formed is the system of intracellular signal transmission. It has been shown that fluorine compounds activate cascades of secondary messengers (Agalakova and Gusev 2011) (Fig. 2). For example, inorganic fluorine compounds activate G-proteins in the liver,

pancreas, and endothelium cells. The adenylate cyclase and phospholipase C enzymes are the main effectors of G-proteins. It has been shown that NaF in the body is capable of forming complexes with metal ions, in particular with aluminum ions AlF_4^-. The AlF_4^- molecule is a structural analog of the phosphate group (PO_4^{3-}) and therefore is capable of acting on ATP- and GTP-converting enzymes involved in many metabolic reactions in the cell. AlF_4^- is able to pass through the cell membrane and interact with the membrane-bound α-subunit of the G-protein, resulting in the formation of the Gα-HDF-AlF_4-complex. This complex activates G-protein with subsequent stimulation of various intracellular signal transduction pathways –protein kinase A, protein kinase C, phosphatidylinositol-3-kinase, etc. (Refsnes et al. 2003).

Inorganic fluorine compounds affect the system of cyclic nucleotides – cyclic adenosine monophosphate (cAMP) and guanosine monophosphate (cGMP). A change in the level of cyclic nucleotides regulates the degree of phosphorylation of the corresponding proteins, which determines the activity and direction of metabolic processes. An increase in these nucleotides has been shown in the heart, liver, kidneys, and adrenal glands. At the same time, the intracellular content of cAMP in the studied organs was increased as a result of the increased activity of the adenylate cyclase enzyme. In addition, under fluoride intoxication, the cAMP/cGMP ratio changes due to a significant excess of cAMP. Non-selective activation of GTP by metallo-fluorides leads to wide dysregulation of functions, including cell signaling, transport, and cytoskeleton integrity (Agalakova and Gusev 2011) (Fig. 2).

Biomarkers of Programmed Cell Death (Apoptosis)

Currently, works have appeared on the role of fluorine compounds in the induction of programmed cell death – apoptosis and necrosis. High fluorine concentrations cause necrosis of hepatocytes (Ghosh et al. 2008), thymocytes (Matsui et al. 2007), erythrocytes (Agalakova and Gusev 2011), leukocytes (Gutiérrez-Salinas et al. 2010), etc. It has been shown that ROS and an increase in the level of intracellular Ca^{2+} are involved in the development of necrosis. Fluorine is an inducer of apoptosis in leukocytes, fibroblasts, alveolocytes, and lung epithelial cells (Karube et al. 2009; Gutiérrez-Salinas et al. 2010). The mechanisms of fluoride-induced apoptosis include (i) an increase in the level of ROS and activation of free radical oxidation (Gutiérrez-Salinas et al. 2010); (ii) damage to mitochondria and activation of the mitochondrial pathway for triggering apoptosis (Matsui et al. 2007; Karube et al. 2009), which requires the expression of pro- and anti-apoptotic genes and de novo synthesis of mRNA, Bcl-2 and p53 (Lee et al. 2008); (iii) an increase in the intracellular level of Ca^{2+} and an increase in the number of cells expressing the marker of apoptosis, annexin V (Matsui et al. 2007); (iv) activation of the caspase cascade, caspases 3, 8, and 9 (Wang et al. 2009); and (v) changes in the activity of intracellular signaling pathways – an increase in the activity of protein kinase C (Refsnes et al. 2003) and MAPK (Karube et al. 2009) (Fig. 2).

Fluoride, in the form of HF, diffuses intracellularly, leading to the induction of apoptosis. Fluoride interacts with Bcl-2 in the mitochondrial membrane, causing cytochrome c (CytoC) release and activating caspase-9 and caspase-3. F induces mitochondrial ROS production, ultimately releasing G-protein-coupled receptor 78 (GPR78) from the endoplasmic reticulum and activating inositol-requiring enzyme 1 (IRE1). Under prolonged endoplasmic reticulum stress, activated IRE-1 induces upregulation of pro-apoptotic proteins like C/EBP homologous protein (CHOP), thus inducing apoptosis. Fluoride activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) by abrogating the inhibiting effect of an inhibitor of nuclear factor kappa B (IκB) and inducing the expression of apoptosis-related genes, such as GRP78, IRE-1, and XBP-1 (Zuo et al. 2018) (Fig. 2).

Alterations in Cell Metabolism

It has been shown that fluorine compounds have a high affinity for some metal ions, which play the role of cofactors in the activity of enzymes of the main metabolic pathways. In most cases, fluoride acts as an inhibitor of enzymes, but sometimes it can stimulate their activity. The mechanisms depend on the type of enzyme, concentration, and duration of fluorine action (Adamek et al. 2005; Agalakova and Gusev 2011). In micromolar doses, fluorine is considered an effective anabolic agent, while millimolar concentrations inhibit various enzymes, including phosphatases. In addition, fluorine ions are able to bind to functional groups of amino acid residues in the active center of enzymes, which also causes their inhibition. In this way, the activity of Na^+,K^+-ATPase is inhibited, which leads to depletion of the level of ATP in the cell and disruption of the cellular membrane potential (Agalakova and Gusev 2011). A decrease in ATP production causes an increase in the level of AMP, ADP, GDP, and inorganic phosphorus in the cell (Fig. 2).

The chronic effect of high concentrations of fluoride also changes the parameters of carbohydrate and lipid metabolism. Thus, experiments on mice showed the development of hyperglycemia by the fourth week of fluoride intoxication (García-Montalvo et al. 2009). At the same time, the level of insulin mRNA was decreased in the β-cells of the pancreas in these mice. Fluorine ions also inhibit the pentose phosphate pathway of glucose oxidation, in particular the enzyme glucose-6-phosphate dehydrogenase (Bergandi et al. 2010).

Fluorine compounds affect the level of phospholipids in the membranes of various organs and blood serum. A significant increase in lysophosphatidylcholine, sphingomyelin, and phosphatidylserine (detergent fraction of phospholipids) and a decrease in phosphatidylcholine and phosphatidylethanolamine in blood plasma, erythrocyte membranes, and cells of various organs (liver, kidneys) have been shown (Wang et al. 2009). Fluoride intoxication is accompanied by a change in the quantitative composition of saturated and polyunsaturated fatty acids – in the lipids of erythrocytes and hepatocytes, increased levels of myristic, oleic, and linoleic acids prevail, while the level of arachidonic acid significantly decreases

(Wang et al. 2009). It is important that changes in the composition of fatty acids accompany the increased activity of phospholipase A2 and indicate the possibility of violation of the synthesis of eicosanoids: prostaglandins, prostacyclins, thromboxanes, and leukotrienes (Ridley and Matsuoka 2009). In addition, changes in the level of membrane phospholipids and saturated and polyunsaturated fatty acids indicate a violation of the structure of cell membranes, i.e., their rigidity increases, and the lipid-protein interactions change, which leads to disruption of the functioning of the transport and cell enzyme systems. In general, approximately 80 proteins, the activity of which is reversibly changed by fluorine ions, are currently known. Almost all of these proteins are involved in basic metabolic processes in the organism (Johnston and Strobel 2020).

Applications to Prognosis

Regardless of the route of entry and/or exposure conditions, fluorine entering the human body has a toxic effect on a whole complex of organs and systems, including the cardiorespiratory, neuroendocrine, and musculoskeletal systems. The study of the effect of fluorine and its compounds on population health is an important task, which makes it possible to outline the ways of prevention and correction of negative effects. The study of the influence of fluorides on the development and course of somatic diseases is promising. The problem of treatment and prevention of the negative effects of fluorides is relevant for general practitioners. The use of sorbents can be an effective way to prevent excessive fluoride ions from entering the organism. A positive effect of certain vitamins (C, B group, PP), calcium and magnesium compounds, and salts of lactic and pyruvic acids in mitigation of fluorine intoxication has been reported.

Mini-dictionary of Terms

- Antioxidants are substances that can prevent chain reactions triggered by free radicals, which are dangerous for the organism.
- Apoptosis is a regulated process of programmed cell death, as a result of which the cell breaks down into separate apoptotic bodies, limited by the plasma membrane. Fragments of a dead cell are usually very quickly phagocytosed by macrophages or neighboring cells, bypassing the development of an inflammatory reaction. One of the main functions of apoptosis is the destruction of defective (damaged, mutant, infected) cells.
- Dental fluorosis (endemic dental fluorosis) is a chronic disease that occurs in areas with excess fluoride in drinking water. The disease develops before teething. In the case of fluorosis, mainly the tooth enamel is affected.
- Intracellular signaling is a chain of sequential biochemical reactions carried out by enzymes, some of which are activated by secondary messengers.

- Lipid peroxidation is a chemical process, a cascade of reactions for conversion of lipids (supplied with food or synthesized in the organism) with the participation of free radicals, i.e., active charged molecules. Since lipids are components of the membranes of all cells, peroxidation reactions can lead to violation of their structure and damage to the cell, which is one of the mechanisms of the pathogenesis of a number of diseases.
- Osteoblasts are cells of mesenchymal origin that produce bone tissue in vertebrates and humans.
- osteoclasts are cells that destroy bone tissue during its rearrangements in vertebrates and humans.
- Oxidative stress is the process of damage and death of human cells as a result of oxidation.
- Reactive oxygen species (ROS) include oxygen ions, free radicals, and peroxides of both inorganic and organic origin. These are usually small molecules with exceptional reactivity due to the presence of an unpaired electron at the external electronic level.
- Remineralization is the process by which previously lost minerals (such as calcium and phosphate ions) are returned to the tooth structure. The degree of loss of minerals in the tooth structure determines the possibility of enamel restoration.
- Transcription is the process of RNA synthesis using DNA as a template occurring in all living cells.
- Translation is the process of protein synthesis from amino acids carried out by the ribosome on the matrix of messenger RNA (mRNA); implementation of genetic information.

Key Facts of Fluorosis

Excessive intake of fluoride with water is the cause of endemic fluorosis. It affects the teeth in the form of the so-called speckled enamel (a defect in the enamel surface of the tooth with a yellowish coating in these areas) and the skeleton of people from birth or early age living in foci of endemic fluorosis. Emissions from industrial production, especially cryolite and superphosphate plants, significantly pollute the environment with fluorine. In this case, vegetation dies, since fluorine destroys chlorophyll. Cattle grazing on contaminated pastures can develop bone fluorosis.

Fluorosis is a very common disease associated with excess fluoride intake during the enamel formation period. Therefore, it is necessary to analyze all factors affecting the occurrence and course of the pathological process. Of interest is not only the fluoride content in drinking water but also the dynamics or its absence when a patient with fluorosis moves to areas with normal fluoride content in water, and it is necessary to take into account the duration of exposure of tissues and organs to fluorides.

Summary Points

Fluoride ions can have a positive effect on the bone system and tooth enamel, but their positive effect is possible only in micromolar doses. At the same time, even at these concentrations, the generation of free radicals and, as a consequence, the activation of MAPK-dependent apoptosis pathways increase in all other organs and systems. Fluoride can cause the simultaneous development of several types of hypoxia. The development of some of the most threatening effects of fluoride is dose-dependent and requires millimolar concentrations. Given the data from the last decade, a question arises of the effectiveness of water fluoridation as a means of prevention of dental diseases. The analysis of numerous studies revealed that fluorine compounds are a cytotoxic factor involved in changes in metabolism, modulation of intracellular signaling pathways, and activation of programmed cell death. In this case, the mechanisms of the physiological or toxic effects of fluorine compounds on the organism depend on their concentration and duration of exposure. Therefore, investigations of the molecular mechanisms of fluoride exposure, despite previous findings, are promising means of elucidation of the general mechanisms of fluoride-associated diseases.

Cross-References

► Biomarkers in the Biomonitoring of Fluoride Toxicity: An Overview

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Biomarkers of Alcohol Toxicity

11

Natalia Kurhaluk and Halyna Tkachenko

Contents

Abstract

The aim of this review is to explore the current understanding of the mechanisms and course of alcohol intoxication, in particular by discussing the classification of various methods of determination of alcohol abuse markers and by reviewing the symptoms of the disease and associated diagnostic markers in clinical and laboratory examination. The chapter presents the current information about examinations performed to confirm alcohol abuse or exclude alternative causes. Treatment of patients and prognosis are presented as well. Biochemical indicators

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with the greatest value for objective laboratory diagnosis of alcohol dependence are reviewed.

Keywords

Alcohol dependence · Diagnosis · Clinical methods · Biochemical methods · Psychological · Instrumental methods

Abbreviations

ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CDT	Carbohydrate-deficient transferrin
CK	Creatine kinase
CNS	Central nervous system
DCM	Dilated cardiomyopathy
EtG	Ethyl glucuronide
EtS	Ethyl sulfate
GABA	Gamma-aminobutyric acid
GGT	Gamma-glutamyl transferase
HPLC	High-performance liquid chromatography
MAO	Monoaminoxidase
MCV	Mean corpuscular volume of erythrocytes
NMDA	N-Methyl-D-aspartate
PEth	Phosphatidylethanol
UA	Uric acid

Introduction

Alcohol is the most commonly consumed toxic substance in the world (Denooz et al. 2019). Approximately 10% of adults in economically developed countries consume alcoholic beverages in excessive quantities. However, alcohol consumption is part of the culture of many nations and may be one of the most significant global social and health problems. Alcohol (ethanol or ethyl alcohol) is an ingredient of beer, wine, and spirits that causes drunkenness. Alcohol is formed when yeast ferments (breaks down without oxygen) the sugars in different foods. Wine is made from the sugar in grapes, beer from the sugar in malted barley (a type of grain), cider from the sugar in apples, and vodka from the sugar in potatoes or other plants (Fernández-Solà 2020). Ethyl alcohol, i.e., a commonly consumed xenobiotic, is one of the factors affecting the redox state of cells in the organism. Although its metabolism takes place mainly in the liver, alcohol as a solvent penetrates easily through cell membranes in most organs consisting of lipid derivatives.

The problems of excessive alcohol consumption and their associated consequences have reached alarming proportions, and alcohol has become one of the most important health risks around the world (Farren and Tipton 1999). Alcohol use contributes more to disease, injury, disability, and premature death than any other risk factor. In addition to damage to their own health, alcohol abuse causes many social problems associated with increased violence and increased road traffic injuries that affect people who do not drink alcohol (Andresen-Streichert et al. 2018).

Chronic alcoholism is caused by regular consumption of alcohol (Kip et al. 2008). This phenomenon is therefore dangerous for the health of the individual himself and is highly detrimental to society. The prevalence of chronic alcohol abuse in the world suggests an urgent need to develop a set of measures aimed at finding new effective markers for monitoring and assessing alcohol consumption. Modern screening methodologies make it possible to identify, with a high degree of certainty, individuals prone to chronic alcohol abuse. Diagnostic methods that allow early identification of chronically dependent individuals and the monitoring of the effectiveness of treatment in those susceptible to alcohol abuse have an important place among such methods (Wurst et al. 2014).

Alcohol as a Silent Universal Killer

Alcohol is one of the most popular products today (González-Reimers et al. 2014). Is it possible to consider alcohol as a dietary element of modern society, since alcoholic drinks of all kinds and brands are in demand by the vast majority of modern people and nearly everyone consumes alcohol one way or another? After all, the only differences are the types of drinks consumed, the reasons for drinking, and the regularity of alcohol consumption (Jellinek 1960; Helander 2003). Despite the debate among experts as to whether alcoholism should be considered a disease, as the beneficial qualities of wine have recently been frequently debated in the scientific literature, the National Institute on Alcohol Abuse and Alcoholism does recognize alcoholism as a disease (Jellinek 1960).

Alcohol and nutrition are closely related (Towiwat and Li 2015), which can lead to eating disorders. Chronic alcoholics do not eat enough food due to the high caloric content of alcohol. It also prevents them from appropriate intake of vitamins and minerals required for health. Importantly, large quantities of alcohol impede or completely stop the digestion of food, as alcohol reduces the secretion of digestive enzymes by the pancreas. Alcohol also interferes with the transport of nutrients into the bloodstream. These disturbances in digestion and absorption over a long period of time can lead to exhaustion. Alcohol is a universal poison that destroys all human systems and organs (Maisch 2016; Piano and Phillips 2014).

Ethanol and its metabolites, e.g., acetaldehyde and acetate, have a direct toxic effect on the myocardium. Patients who drink alcoholic drinks in large quantities over a longer period of time may also develop deficiencies of certain vitamins (B_1), micronutrients (selenium), and electrolytes (magnesium, phosphorus, potassium), which may additionally lead to heart muscle damage. Toxic effects of ethyl alcohol

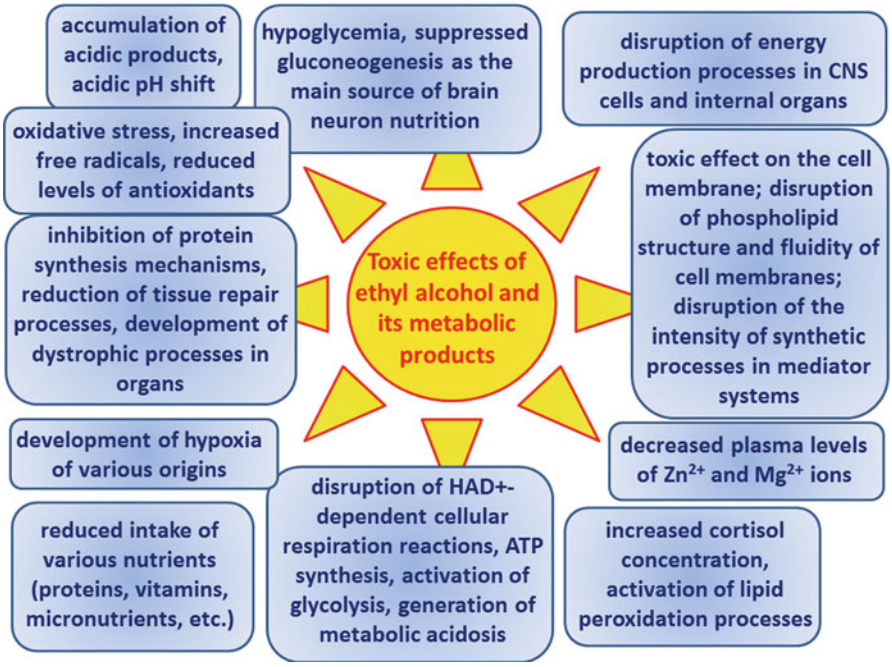

Fig. 1 Toxic effects of ethyl alcohol and its metabolic products

and its metabolic products were shown in Fig. 1. Some alcoholic drinks are contaminated with lead or cobalt, although trace amounts of these compounds do not independently cause alcoholic cardiomyopathy (Fernández-Solà and Nicolás-Arfelis 2002).

The alcohol dependence, withdrawal syndrome, and tolerance are related to the malfunctioning of various biochemical and physiological processes, many of which can be seen as primary pathogenetic factors in the development of the disease (Shader 1994). Such factors include increased production of acetaldehyde from ethanol in the liver in the presence of excessive alcohol, damaging effects of alcohol on brain cell membranes, formation of morphine-like alkaloids in brain tissue, inhibition of RNA and protein synthesis in the brain (Cowen and Lawrence 1999), changes in the functional activity of the brain neurotransmitter systems, and membrane regulatory proteins involved in nerve impulse transmission in a number of neurotransmitter systems, such as monoaminoxidase, GABAergic, glycinergic, and glutamatergic, the latter involving NMDA receptors (Kalluri et al. 1998). These neurotransmitter systems are also actively involved in the formation and maintenance of pathological dependence and the development of tolerance. Considering that alterations in neurotransmitter systems are essential to the formation of alcohol dependence, it can be assumed that neurochemical mediation disorders should be looked for in this system and at this level.

Direct and Indirect Markers of Alcohol Use

The difficulties in diagnosing chronic alcohol misuse have three components. The first is the failure to recognize or underestimate alcohol dependence, the second is the overdiagnosis or misdiagnosis in the presence of other etiologies, and the third is the misdiagnosis of the stage or form of alcohol dependence itself (Wurst et al. 2015). Inadequate diagnosis of alcohol dependence is largely related to the fact that chronic alcohol abuse can vary clinically, from asymptomatic latent forms to severe prognostically adverse forms with very high mortality (Wurst et al. 2014).

Laboratory markers of alcohol use are divided into groups of direct and indirect biomarkers (Kip et al. 2008; Thon et al. 2013). Biomarkers differ in the mechanisms of their pathological elevation, which largely determines their analytic specificity, the alcohol dose and duration of alcohol consumption required to elevate biomarker concentrations, and the half-life (or metabolic period) in the individual, which is an important variable in assessing the diagnostic significance of a marker for differentiating chronic abuse and allowing early detection of relapse (Helander 2003; Alladio et al. 2017).

Clinical Methods

The CNS is one of the most sensitive structures to the toxic effects of ethanol. The spectrum of ethanol effects on the CNS is quite wide. In small doses, alcohol has a depressant effect. When large doses of ethanol are consumed, a more widespread inhibition of a large number of different CNS structures develops, leading to disorganization and disturbance of highly integrated processes.

Among the many visceral lesions that affect overall life expectancy in alcoholism, liver pathology takes the lead. In addition to being the main target for ethanol, the liver is also the main organ responsible for homeostasis and energy metabolism in the body.

Another important aspect of the effects of ethanol on the body is damage to the skeletal musculature, which occurs in about 40–60% of cases of alcohol intoxication. This process is accompanied by the development of acute alcoholic myopathy. Clinically, this is manifested by painful swelling of muscle tissue, myoglobinuria, increased serum creatine kinase activity, necrosis, and acute renal failure. The methods for diagnosing alcohol dependence are shown in Fig. 2.

The manifestation of clinical signs of alcohol dependence is closely related to the stage of the disease, some manifesting only at certain stages of the disease and others presenting at all stages of the disease, varying according to the degree of manifestation of the disease. Hence, different clinical variants of the course of the disease are distinguished, depending on the disease progression, form of alcohol abuse, premorbid personality traits of the patient, and accompanying somatoneurological and mental disorders presented in one of the early works (Jellinek 1960).

The syndromology of alcohol dependence according to the classification proposed by Shader (1994) is as follows: development of tolerance to alcohol – a

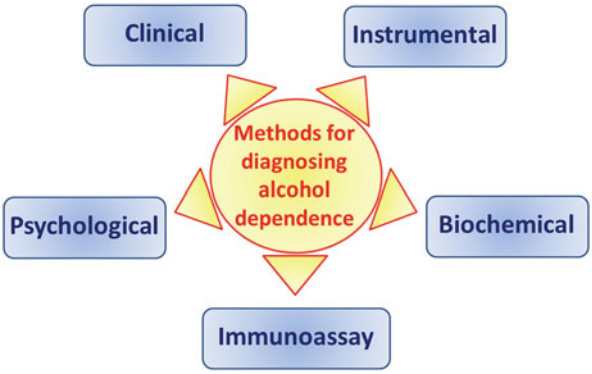

Fig. 2 The methods for diagnosing alcohol dependence

gradual increase in the dose causing intoxication, a markedly weakened effect of habitual doses, and relatively normal behavior at such a concentration of ethanol in the blood as would cause significant disturbance in a nondrinker or low-drinker; appearance of alcohol withdrawal syndrome; need for alcohol to relieve withdrawal syndrome; inability to regulate the alcohol dose and duration of alcohol withdrawal; inability to perform household and work duties properly; repeated drinking in risky situations (e.g., driving or before work that requires good coordination and quick reactions); complete or partial cessation of professional activities and narrowing of interests; committing illegal acts while intoxicated and complicating relationships with others; and continued drinking despite being aware of its adverse consequences.

Instrumental Methods

Prolonged alcohol abuse causes certain structural and functional changes in the brain, depending on many factors (age, sex, duration of alcohol abuse, premorbid personality traits, etc.). Anamnesis findings are of importance here, as traumatic brain injury, poor nutrition, liver and kidney disease, use of other pharmacological agents, and hereditary factors are important. Chronic alcohol abuse leads to loss of brain mass and, in particular, to a deficit in the mass of mammillary bodies (Charness et al. 1988) in Wernicke's encephalopathy (Wernicke-Korsakoff syndrome), cortical degeneration, increased spaces between cerebral surface sulci, and dilated cerebral ventricles (Sivolap 2005; Fama et al. 2012).

Classification of Biological Markers

Secretory enzymes are synthesized in the liver. Then they are secreted into the blood plasma and exert their action there. This group includes blood coagulation factors, cholinesterases, and ceruloplasmin. Indicator (cytolytic) enzymes (AST, ALT, GDH,

lactate, malate dehydrogenases, etc.) allow estimating the degree of hepatocyte destruction. Membrane-bound excretory enzymes that are elevated in the blood during cholestasis include GGT, alkaline phosphate, leucinaminopeptidase, and 5-nucleotidase.

The polymorphism of the enzymes allows each biochemical reaction to be carried out with such specificity and velocity that optimally provides a response of the organism to various stimuli, in turn, determining the optimal specificity and velocity of detoxification of toxicants entering or forming in the organism under the action of physical, chemical, biological, or mental stimuli. Moreover, the more functionally important the enzyme, the wider its isoenzyme (structural) spectrum.

The classification of known biological markers of ethanol use is based on three parameters (Fig. 3). The first group includes markers depending on the physico-chemical properties of ethanol and acetaldehyde – increased red blood cell mean corpuscular volume and decreased filterability, thrombocytopenia, detection of alcohol in the blood, increased activity of alcohol dehydrogenase (ADH) and the microsomal ethanol oxidation system, decreased aldehyde dehydrogenase (ALDH) activity, levels of blood acetaldehyde, etc. The second group consists of markers depending on metabolic disorders caused by ethanol, e.g., hyperlipemia, total hypercholesterolemia, hypertriglyceridemia (elevated high-density lipoprotein cholesterol levels), ketoacidosis and hyperuricemia, increased JgA/transferrin ratio, increased L-amino-H-lactic acid/leucine ratio, decreased urea, nitrogen levels with

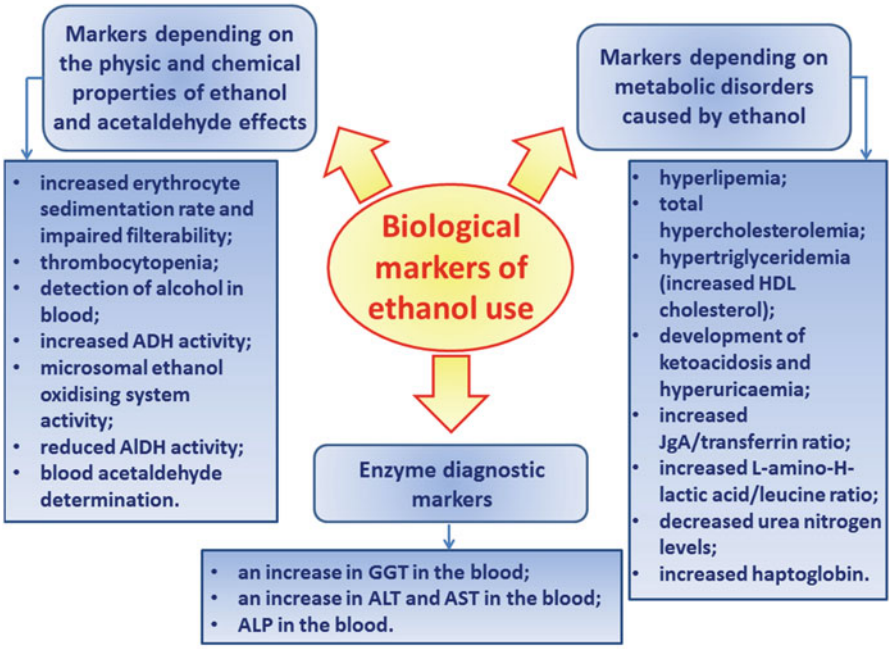

Fig. 3 The classification of biological markers of ethanol use

normal creatine and increased haptoglobin, etc. The third group of enzyme markers includes gamma-glutamyl transferase, alanine and aspartate aminotransferases, and alkaline phosphatase (Farren and Tipton 1999; Denooz et al. 2019; Palmer et al. 2019).

It should be noted that the search for biochemical markers of chronic alcohol use indicates that no single biochemical test taken in isolation is sufficiently reliable for the diagnosis of alcohol dependence (Dinevski et al. 2011). However, the simultaneous use of several biochemical assays may serve as a reasonably good support criterion for objective diagnosis (Aertgeerts et al. 2001).

Biochemical Methods

It should be noted that ethanol is degraded in the organism to acetaldehyde and then to acetic acid, which is further degraded to water and carbon dioxide through incorporation into biochemical reactions in various cycles. It is during the steps of converting ethanol to acetaldehyde and acetaldehyde to acetic acid that the enzymes ADH and ALDH are activated. 95% of acetaldehyde is produced and oxidized in the liver and is the basis of the clinical manifestation of alcohol withdrawal. Therefore, these two enzymes involved in the metabolism of these highly active chemicals, i.e., ethanol and acetaldehyde, are most indicative in the diagnosis of alcohol dependence (Pohanka 2016).

The metabolism of alcohol in the organism is primarily associated with the synthesis of fatty acids and lipids mediated through acetyl-CoA synthetase reactions with oxidation of ethanol. The generation of fatty acids is accompanied by the formation of a reduced form of NAD ($NADH_2$) in cells, leading to a predominance of fatty acid synthesis over fatty acid breakdown. Hence, ethanol abuse leads to increased triglyceride plasma levels (Klop et al. 2013). Moderate hypertriglyceridemia is one of the most consistent features of alcohol dependence.

The determination of the creatine kinase (CK) activity and changes in its isoenzyme profile is of great diagnostic value as an indicator of the integrity of certain tissue structures. It is known that CK plays a particularly important role in the development of polyneuropathies and myopathies associated with alcohol addiction (Segal et al. 2009).

Research is currently being carried out worldwide on diagnostic markers of alcohol consumption. The enzymes tested in the laboratory [aspartate aminotransferase (AST) and alanine aminotransferase (ALT)] are synthesized by hepatocytes and are not specific markers of alcohol use.

It is possible to determine the ethyl alcohol content in body fluids (blood and urine) less than 12 h after alcohol consumption. Before this time, alcohol can be detected, as it is excreted unchanged in urine, sweat, and exhaled air. The rest of the ethanol ingested is absorbed in the stomach and intestines. Most of the absorbed alcohol is oxidized in the liver by alcohol dehydrogenase, aldehyde dehydrogenase, and microsomal ethanol-oxidizing enzymes to form acetaldehyde. Acetaldehyde is a

key factor in the development of many of the pathological conditions associated with alcohol use.

The direct markers of alcohol use include its breakdown products in the organism, such as fatty acid ethyl esters, phosphatidylethanol, ethyl glucuronide, and ethyl sulfate. The period of their detection in different body fluids can range from 8–12 h to 5–7 days. At present, the direct markers of alcohol use are very rarely used in clinical practice (Mundle et al. 1999). The currently available markers and methods to evaluate alcohol consumption are indirect and suboptimal or rely on self-report. The direct metabolites of alcohol, i.e., phosphatidylethanol (PEth), ethyl sulfate (EtS), and ethyl glucuronide (EtG), are known to improve diagnostic accuracy, as shown by Nguyen et al. (2018), and are used to monitor alcohol abstinence in individuals who are mandated to abstain (Reisfield et al. 2020).

The indirect marker group includes a wide range of indices varying widely in their analytical characteristics and diagnostic significance (Hietala et al. 2006). Lowery et al. suggests that phosphatidylethanol is a direct alcohol biomarker for identifying alcohol misuse. It carries several advantages over other alcohol biomarkers, including a detection half-life of several weeks and little confounding by patient characteristics or organ dysfunction (Lowery et al. 2018).

Quantitative assessment of liver enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), is used. Elevated AST levels are often the first indicator of a hepatocyte response to drugs and toxic agents. AST and ALT levels reflect generalized liver cell damage or increased cell membrane permeability, whether of alcoholic or nonalcoholic origin. Both enzymes enter the bloodstream when cell membranes are damaged and are present in many tissues. AST and ALT levels (half-life of 17 and 47 h, respectively) are elevated in alcohol abuse, but because of their very low sensitivity and specificity, these markers cannot be considered as independent indicators of chronic abuse (Whitfield and Martin 1985; Conigrave et al. 2002; van Beek et al. 2013; Adkins et al. 2017).

These traditional indirect markers of alcohol are inexpensive and easy to assess but not sufficiently reliable and have a number of important limitations due to their insufficient sensitivity, diagnostic specificity, etc. These markers give false-positive results in some liver diseases, not always those associated with alcoholism or certain medications (Adkins et al. 2017). Alcohol biomarkers include tests indicative of acute or chronic alcohol consumption (state markers) and markers of a genetic predisposition to develop alcohol dependence after chronic exposure (trait markers), as suggested by Helander (2003).

Gamma-glutamyl transferase (GGT) is a membrane glycoprotein (enzyme) that catalyzes the transfer of a gamma-glutamyl residue from glutathione to various protein acceptors. The level of the increase in serum GGT in response to different amounts of alcohol and at different durations of abuse may vary considerably from patient to patient. As shown by van Beek et al. (2014), the epidemiological association of alcohol intake with GGT is at least partly explained by genetic pleiotropy. First of all, GGT is an indicator of chronic consumption of high doses of alcohol but remains within normal limits in heavy drinkers, nondrinkers, and drinkers but not abusers, if they do not have a comorbid liver disease. Blood levels of GGT are used

as a marker for (heavy) alcohol use. The role of GGT in the antioxidant defense mechanism, which is an important part of normal metabolism, allows an assumption of a causal effect of alcohol intake on GGT. The half-life of GGT is 14–26 days, and the concentration of the enzyme in serum usually returns to normal 4–5 weeks after cessation of alcohol use (Conigrave et al. 2002; van Beek et al. 2014). GGT may rise nonspecifically in pancreatic diseases, type II diabetes, obesity, hypertension, or myocardial infarction and is associated with risk factors for nonalcoholic fatty liver disease, such as dyslipidemia, insulin resistance, hypertension, chronic obstructive pulmonary disease, and renal insufficiency (Loomba et al. 2010). Thus, the main disadvantage of this biomarker is its low specificity.

In recent years, a new marker – carbohydrate-deficient transferrin (CDT) – has become increasingly common around the world. According to literature data, CDT has the best analytical performance of any marker in the laboratory evaluation of chronic alcohol abuse (Arndt et al. 2006). Specific increases in CDT are observed in individuals consuming at least 50–80 g of alcohol for at least 7–10 days, which allows chronic alcohol abuse to be diagnosed in a laboratory evaluation. In the majority of patients with liver disease, however, the CDT level remains within the normal range, which compares favorably with GGT, ALT, and AST (Conigrave et al. 2002; Bortolotti et al. 2006, 2018; Hietala et al. 2006). Some chronic liver diseases can cause false-positive results. However, according to various studies, this marker has the highest specificity and sensitivity.

Transferrin (Tf) is a serum protein synthesized mainly by hepatocytes and is the main carrier of iron in the body. The transferrin molecule consists of three structural domains: a polypeptide chain, two independent iron ion binding sites, and two N-glycan complexes (Stibler et al. 1978). All three domains are highly variable, providing substantial microheterogeneity in transferrin molecules (Gomme et al. 2005). For the diagnosis of chronic alcohol abuse, the greatest importance is ascribed to the variability of transferrin molecules reflected in changes in its N-glycan chain structure. The N-glycan chains in the transferrin molecule structure may differ in the degree of branching to form bi-, tri-, and tetra-antennae. Each antenna may terminate in a sialic acid molecule carrying a negative charge.

Depending on the number sialic acid residues bound to the antennae of the carbohydrate chains, up to nine variants (isoforms) of transferrin are detected in human serum, ranging from asialo- to octasialotransferrin (Mårtensson et al. 1997). The percentage of these isoforms in serum is strictly orderly. In chronic alcohol abuse, glycosylation of transferrin is impaired, leading to a change in the percentage of its isoforms to an increased level of low-sialylated variants, also referred to as carbohydrate deficient, or CDT (Liang et al. 2021). Since the identification of different forms of transferrin, their determination has been done with different analytical methods. The poor analytical performance of a particular method can significantly reduce or completely devalue the diagnostic value of CDT (Bortolotti et al. 2006). Therefore, the practical use of the CDT marker should be implemented only by those diagnostic methods whose characteristics fully satisfy the criteria for their selection (Bortolotti et al. 2018). To date, a wide range of methods have been

developed for determining CDT levels, including indirect and direct immunonephelometry, high-performance liquid chromatography (HPLC), and capillary electrophoresis (Lanz et al. 2004).

Immunonephelometric techniques do not allow complete separation of low-sialyte isoforms from high-sialyte isoforms of transferrin and do not allow identification of genetic variants of transferrin. This negatively affects the analytical specificity of the marker and increases the risk of false-positive results. Due to the poor efficacy of this approach, immunological methods have been excluded from the diagnostic mechanism of CDT assessment. Separation methods, such as HPLC and capillary electrophoresis, are devoid of the limitations of immunological methods, allowing detection, imaging, and quantification of CDT isoforms as a total of all carbohydrate-deficient fractions, as well as isolation of disialotransferrin. In addition, separation methods allow the detection of possible genetic variants of transferrin, thereby minimizing the risk of misinterpretation of test results (Stibler et al. 1980; Arndt et al. 2006).

Disturbances in the functional state of brain neurotransmitters under the influence of ethanol are one of the most important signs of alcohol intoxication. Alcohol alters not only the synthesis, release, and metabolism of individual neurotransmitters but also the process of their reception. Monoaminoxidase (MAO) as an indicative biochemical marker of neurotransmitter disorders in alcohol dependence is important in the development of a number of neuropsychiatric disorders and substance abuse disorders, because it participates in the formation of the dopamine vicious cycle. The individual constancy of MAO levels, specificity of inhibition of its activity by ethanol, independence from changes in the withdrawal, psychosis, and depression states and treatment confirm the hypothesis about the hereditary determination of MAO levels and allow using this criterion as a genetic marker with high accuracy of alcohol dependence diagnosis (Comings and Blum 2000).

In recent years, much attention in the mechanisms of ethanol action on the CNS has been given to the GABAergic system and GABA itself, which is the main inhibitory mediator in the brain. Some biochemical and behavioral effects of ethanol are thought to be related to the increased activity of the GABAergic inhibitory system and to affect the ability of GABA to bind to specific receptors and alter the metabolism of the mediator, increasing synaptic transmission of nerve impulse in GABAergic neurons. The concentration-dependent effects of ethanol are thought to be related to different mechanisms of altering the protein-lipid arrangement of the GABA receptor/chlorine channel complex (Caputo and Bernardi 2010).

The multiple and varied effects of ethanol on the CNS are related to disturbances in the metabolism of biogenic amines, especially dopamine. The dopaminergic system of the brain plays a prominent role in the genesis of various psychiatric and motor disorders and is involved in the control of brain reinforcing mechanisms, in particular hypothalamic self-stimulation mechanisms. The dopaminergic neurons in the limbic and prefrontal cortex are most vulnerable to the effects of alcohol. This suggested that genetic differences in ethanol preference in animals might be based on alterations in dopaminergic neural impulse transmission (Di Chiara 1997).

The conversion of ethanol is known to produce more toxic products with a pronounced effect on the production of red blood cells, which clinically manifests itself after several days or weeks of heavy alcohol consumption. The most common manifestation is an increase in the size of the red blood cells (their mean corpuscular volume) and the appearance of a moderately pronounced anemia. At the same time, the hardness, viscosity, and electrical conductivity of the erythrocyte membrane increase with alcoholic heart damage, and the relationship between the electrical characteristics of erythrocytes and myocardial bioelectrical activity has been revealed. These data allowed the determination of the mean corpuscular volume (MCV) of erythrocytes to be considered as an effective biomarker together with other parameters of biochemical analysis (Pavanello et al. 2012).

Alkaline phosphatase (ALP) activity increases sharply in alcohol poisoning with chronic alcohol abuse (Hamarneh et al. 2017). The next marker is uric acid (UA), which is a product of the metabolism of purine bases that are part of complex proteins, i.e., nucleoproteins, and is excreted by kidneys. This parameter is associated with secondary gout seen in acute alcohol intoxication (secondary "alcoholic gout"). Of some interest in long-term alcohol dependence is the increased MC level and ketoacidosis (Towiwat and Li 2015).

A study of the content of ferritin in the tissues of alcohol-dependent patients demonstrated that the absolute and relative content of tissue ferritin in this group of patients was significantly higher and the cause of the change in the concentration of ferritin in tissues in alcohol dependence is the activation of the free radical mechanism of tissue damage (Lorcerie et al. 2017).

The content of ceruloplasmin, i.e. the copper-containing glycoprotein that acts as a free radical scavenger, is significantly impaired in alcohol addiction. This affects the metabolism and concentration of copper, measured by ceruloplasmin value in humans. This is associated with the fact that alcohol addiction causes serious metabolic changes, including macro- and microelement imbalances (Shibazaki et al. 2017).

Research conducted in recent years has shown that the level of mental and neuronal disturbances in alcohol-dependent patients is closely related to the role of melatonin (Kurhaluk and Tkachenko 2020). There is evidence that even a single alcohol drink can cause a decrease in the production of this hormone in the body. It has been pointed out that mental and emotional disorders in alcoholics caused by prolonged periods of insomnia are important deterrents and can be compensated by melatonin administration in different doses as part of the therapy. This hormone, which is limited or significantly diminished in this type of disorder, significantly improves prognosis as part of therapy (Kurhaluk 2021). The base of any alcoholic drink is ethyl alcohol. Ethyl alcohol itself is a highly toxic poison. Therefore, no matter whether it is consumed in a low-alcohol or strong drink, it has a detrimental effect on all internal organs. Moreover, frequent consumption of alcohol is addictive, which leads to alcoholism. The substantial damage to the melatonin-related metabolic pathway of this hormone in alcohol-dependent patients and in animal models indicates the possibility of its involvement in the correction of these conditions, as found in some studies.

Immunochemistry Tests

Immunochemistry tests have been developed to diagnose diseases of the nervous system by measuring the autoantibody titer to brain-specific antigens determined in blood and cerebrospinal fluid. This mechanism is linked to indicators of the degree of destruction of nervous tissue through specific receptors of brain cells that are involved in the autoimmune mechanisms of the diseased organism. In relation to alcohol dependence, researchers have observed a similar pattern of change in immune status as in any disease where there is destruction of nerve tissue. Therefore, a possibility considered to be more promising in terms of diagnosis is the isolation of individual (or a group of) antigens that are directly relevant to the pathogenetic mechanisms of destructive or metabolic abnormalities in brain tissue (Erickson et al. 2019).

Immunoassay diagnosis of alcohol dependence based on autoantibody levels to opioid receptors is considered a promising research opportunity. Based on the understanding of the underlying mechanisms of alcohol dependence, the opioid system plays one of the major roles in the pathogenesis of this pathology. Studying the dependence of the degree of damage to the structure and functions of CNS opioid receptors exposed to ethanol and the nature of alcohol addiction symptoms on the interaction of the opioid system and ethanol according to their behavioral effects and pharmacological effects is of high priority. It has been reported that ethanol binds predominantly to one or more opioid receptors and modulates their effects, which suggests their polymorphism and involvement in ethanol effects. The main cause of the impairment of catecholamine metabolism in alcoholism is an imbalance in the system of endogenous opioid neuropeptides (Di Chiara 1997; Caputo and Bernardi 2010).

Alcoholic Cardiomyopathy

Alcohol abuse is one of the most common causes of acquired dilated cardiomyopathy (DCM), i.e., a dilated cavity and reduced contractile function of the left ventricle (LV) with nonischemic origin and next possible involvement of other parts of the heart (Mirijello et al. 2017). The development of these changes in a patient with a proven history of excessive alcohol consumption has been termed alcoholic cardiomyopathy (ACMP). Broadly speaking, the term can also refer to a wide range of cardiovascular disorders that develop in the context of acute or chronic alcohol intoxication (CAI) and are considered to be part of what is known as alcoholic heart disease (Fernández-Solà 2020).

The prevalence of ACMP among those with dilated DCM can range from 23% to 66%, depending on the level of alcohol consumption in the population, if consumption of more than 80 g of ethanol per day for more than 5 years is taken as a diagnostic criterion, but the prevalence of this condition can be very different when other criteria of alcohol abuse are used (Guzzo-Merello et al. 2014). However, when other criteria for alcohol abuse are used, the rate of this disease may be very different

(Iacovoni et al. 2010). Generally, the prevalence is higher in middle-aged males (Fernández-Solà and Nicolás-Arfelis 2002; Rehm et al. 2017).

Clinical evidence of the toxic effects of ethanol on the myocardium is the increase in troponin levels that can accompany alcoholic withdrawal (Patel et al. 2001). The mechanisms of alcohol-induced myocardial damage are manifold: disruption of protein synthesis, changes in calcium currents and myofilament sensitivity to it, increased oxidative stress, shortened action potential, as well as disconnection of contraction and excitation processes due to impaired intracellular signal transduction (Waszkiewicz et al. 2013), intracellular signal transduction (Leibing and Meyer 2016), induction of inflammation (González-Reimers et al. 2014), and glycogen deposition. Exposure to ethanol leads to disruption of cardiomyocyte repair and replication mechanisms, which in turn enhances cardiac muscle remodeling (Ai et al. 2020). Increased apoptosis mechanisms involve Bax and Bcl-2, whose expression is significantly increased (Piano and Phillips 2014). In rats consuming high doses of alcohol, a clinically significant increase in the activity of caspase-3, a marker of apoptosis, has been reported (Rodriguez et al. 2015).

In recent years, studies in animal models have revealed additional signs of mitochondrial damage in the form of changes in their membrane potential and the activity of respiratory chain enzymes, as well as reduced ATP biosynthesis (Steiner and Lang 2017). A decrease in superoxide dismutase, glutathione metabolites, and malonic dialdehyde as well as lipid peroxidation and increased formation of free radicals and reactive oxygen species were observed. The activity of aldehyde dehydrogenase-2 in mitochondria, which converts toxic acetaldehyde into acetate, may play a special role, and its overexpression allows preventing the consequences of acute and chronic alcohol damage (Palmer et al. 2019; Mansouri et al. 2018; Liu et al. 2018).

Individual susceptibility to ACMP may be expressed by features of myocardial structure or alcohol metabolism (Maisch 2016). Some genetic polymorphisms of enzymes associated with ethanol conversion (alcohol and aldehyde dehydrogenase) are associated with an increased risk of ACMP (Zhu et al. 2017). More frequent detection of point mutations has been reported. The presence of mitochondrial DNA point mutations in patients with this disease is reported, although these changes may also be related to the harmful effects of alcohol (Zapico and Ubelaker 2013).

Laboratory markers of heavy drinking in persons with cardiomyopathy include increased γ-glutamyl transpeptidase (GGT), aspartate and alanine aminotransferases, with a predominance of the former, macrocytosis, transferrin, and a defective form of transferrin that is the most specific test (Bortolotti et al. 2018). Detection of fatty acid ethyl esters in erythrocytes and serum is relevant only on the first day after alcoholic beverage intake and is less applicable for detection of chronic forms of alcoholism (Maisch 2016). Determination of specific GGT fractions may suggest the presence of withdrawal (elevated m-GT and s-GT) or continued drinking in a majority of patients (Franzini et al. 2013). Increases in biomarkers of CHF (natriuretic peptides) and myocardial damage (troponin) are possible (Maisch 2016).

Conclusions

All biochemical tests taken in isolation are not sufficiently reliable or informative for diagnosis. However, there are no sufficiently simple, objective, and easily reproducible tests for diagnosing alcohol dependence and assessing the patient's condition at any stage of the disease. No study of alcohol dependence and evaluation of the patient's condition at different stages of the disease have been carried out to date. However, the simultaneous use of several biochemical indicators can serve as an auxiliary criterion for the objective diagnosis of alcohol abuse. Biochemical parameters of ADH and ALDH activity and their correlation, CDT, CK isoenzymes, MAO, and GABA are the most specific for alcohol addiction diagnostics today. Highly specific test systems have been developed for these indicators, which allow diagnosing alcohol dependence not only in the acute period but also in long-term remission.

Prospects for Further Research

Many steps are being taken to reduce morbidity and mortality from alcohol-related diseases. For example, reductions in alcohol consumption should lead to lower incidence of ACMP in the near future. The key to better understanding the mechanisms of alcohol dependence and the development of specific drugs for the treatment of this disease is the study of translational animal models, in which reduction of apoptosis activity in the myocardium has been shown to be beneficial. New therapeutic strategies for the treatment of ACMP involve the use of myocardial growth and regulatory factors, as well as the stimulation of cardiac muscle regeneration and repair processes. Suppression of the activity of signaling pathways responsible for hypertrophy and cardiomyocyte death seems promising.

When discussing the conditions in which alcohol pathology develops, several elements such as social, psychological, and biological factors have to be kept in mind, because today we can speak with certainty about the biological predisposition of an individual to develop alcohol addiction. Epidemiological studies are also very important, with broad, voluminous objectives – primarily sociopsychological and clinical. The social spectrum of factors in epidemiological research includes the economic, legal, cultural, and ethnic aspects of the group in focus.

Applications to Prognosis

The studies presented in the literature on alcohol toxicity markers demonstrate the difficulty of verifying the problem under investigation, despite the seeming simplicity of the diagnostic criteria. This may be the likely reason for the underestimation of the prevalence of this disease. The study of the effect of alcohol and its compounds on population health is an important task, which makes it possible to outline the ways of prevention and correction of negative effects. A more detailed study of the

mechanisms of ethanol tissue pathogenesis, as well as genetic predisposition, allows new diagnostic and therapeutic methods to be developed and studied, with a possible personalized approach to the management of individual patients in the near future. At the same time, it should be remembered that the most effective method of prevention and treatment of this disease is to reduce alcohol consumption, which is crucial both for the individual patient and at the population level.

The steady growth of alcoholism raises the problem of further deepening of knowledge in the field of its etiopathogenesis, diagnosis, prevention, and treatment. The relevance of the problem of alcoholism has recently increased due to the epidemiological and social danger of this disease. The significance of the problem of alcoholism is constantly important for theoretical and practical medicine. Alcohol is capable of causing damage to most organs, it contributes to the development of more than 60 different diseases and therefore makes a significant contribution to morbidity and mortality. Alcohol can cause damage to most organs, contributes to the development of more than 60 different diseases, and contributes significantly to morbidity and mortality.

Mini-dictionary of Terms

- **Wernicke's encephalopathy (Wernicke-Korsakoff syndrome)** is a degenerative brain disorder caused by the lack of thiamine (vitamin B1). It may result from alcohol abuse, dietary deficiencies, prolonged vomiting, eating disorders, or the effects of chemotherapy. B1 deficiency causes damage to the thalamus and hypothalamus.
- **Hypertriglyceridemia** is a condition in which triglyceride levels are elevated.
- **Polyneuropathy** is a simultaneous malfunction of many peripheral nerves throughout the body caused by infections, toxins, drugs, and other disorders.
- **Dyslipidemia** is an abnormal amount of lipids in the blood.
- **Microheterogeneity** is a variation in the chemical structure of a substance.
- **False-positive result** is a result that indicates that a given condition is present when it is not.
- **Dilated cardiomyopathy (DCM)** is defined as left ventricular chamber dilation with decreased systolic function in the absence of coronary artery disease or conditions that impose a chronic pressure overload.

Key Facts About Alcohol

Approximately 20% of ingested ethanol is absorbed in the stomach and the remaining 80% is absorbed in the intestine.

Only 5% of ethanol is excreted unchanged in the urine, sweat, and exhaled air, where it can be detected within a few hours (less than 12) after consumption.

Ninety to ninety-five percentage of absorbed alcohol is oxidized in the liver by alcohol dehydrogenase, aldehyde dehydrogenase, and microsomal ethanol-oxidizing enzymes to form acetaldehyde.

Acetaldehyde is a key factor in the development of many pathological conditions associated with alcohol use.

Summary Points

This chapter reviews methods for objective diagnosis of alcohol dependence and for monitoring its course. The methods are divided into clinical, biochemical, psychological, and instrumental. Only at complex use of different methods it is possible to draw objective conclusion about condition of patient, prognosis of course of illness, and stagnation. It is only through a combination of different methods that an objective conclusion can be drawn about the condition of the patient. The search for reliable criteria for diagnosing systematic alcohol use and clinical and biological correlations that are sufficiently universal and relevant for diagnosis is an urgent task of modern narcology. For this reason, this chapter attempts to provide an analytical review, including a description of the main diagnostic methods for alcohol dependence. It should be said that by now, apart from the methods considered below, there are a sufficient number of other techniques and tests which cover many fields and sections of medicine – genetics, immunology, immunogenetics, neurobiology, etc.

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Biomarkers of Liver Injury due to Toxic Agents: Progress, Current Applications, and Emerging Directions

12

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Contents

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Abstract

The liver is unusually susceptible to toxicant-induced injury due to its unique physiology and biochemistry, but current liver injury biomarkers have limited value beyond detection of the injury and the resulting impact on liver function. Better biomarkers are needed for (1) diagnosis and determination of etiology, (2) prognosis, and (3) preclinical assessment of the hepatotoxic liability of new drugs and xenobiotics. A number of biomarker candidates that may meet these needs have been identified over the last 10 years. In addition, several biomarkers with mechanistic importance have been proposed. In this chapter, we will briefly review current liver biomarkers and discuss the most popular emerging biomarker candidates. We will also discuss strengths and weaknesses of each and what work remains to be done to move the field forward.

Keywords

Acylcarnitines · Alanine aminotransferase · Acetaminophen · Acute liver failure · Bile acids · Biomarkers · Drug-induced liver injury · Factor V · Glutamate dehydrogenase · Hepatotoxicity · High-mobility group box 1 · Keratin 18 · Lactate dehydrogenase · MicroRNA-122 · Pyrrolizidine alkaloids

Abbreviations

ADH2	Aldehyde dehydrogenase 2
AFP	Alpha-fetoprotein
ALDH1A1	Alcohol dehydrogenase 1A1
ALF	Acute liver failure
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
APAP	Acetaminophen
AST	Aspartate aminotransferase
DILI	Drug-induced liver injury
FABP1	Fatty acid-binding protein 1
FBP1	Fructose-1,6-bisphosphatase 1
FV	Factor V
GGT	Gamma-glutamyl transferase
GLDH	Glutamate dehydrogenase
HMGB1	High-mobility group box 1 protein
INR	International normalized ratio
K18	Keratin 18
LDH	Lactate dehydrogenase
Lect2	Leukocyte cell-derived chemotaxin 2
miR-122	MicoRNA-122
OPN	Osteopontin
PT	Prothrombin time

Introduction

The liver is highly susceptible to injury caused by xenobiotics owing to its unique blood supply and biochemistry (Fig. 1). Most ingested compounds absorbed from the intestinal lumen travel directly to the liver vasculature through the hepatic portal vein and enter the parenchymal cells of the liver – the hepatocytes – through transporters in the cell membranes. Once inside, enzymes highly expressed in the hepatocytes process those compounds, storing useful nutrients (e.g., glucose) or packaging them for distribution to the body (e.g., triglycerides), while simultaneously preparing potentially harmful substances (e.g., drugs or toxins) for elimination through renal and intestinal excretion. This phenomenon of "first-pass metabolism" is critical for proper nutrient utilization and elimination of toxicants before they can reach the rest of the body. However, it also exposes the liver to higher concentrations of exogenous compounds than those seen by cells in other organs. In addition, some of the enzymes that process those xenobiotics actually make the compounds more reactive, resulting in collateral damage to the cells through reactions with proteins and DNA. As a result, numerous toxins and toxicants, ranging from the amatoxins and phallotoxins in certain species of fungi, to carbon tetrachlo-

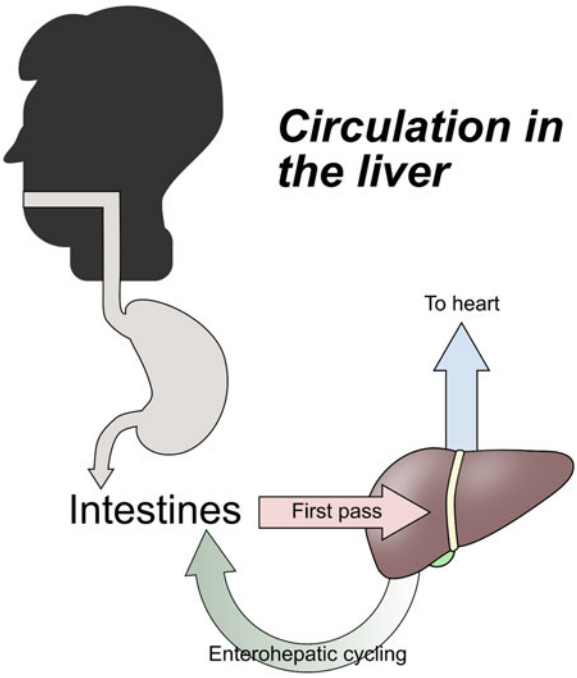

Fig. 1 Hepatic circulation. Ingested compounds travel to the small intestines, where they are absorbed into the blood. Venous blood from the intestines then carries those compounds to the liver, where they are metabolized. Some compounds or their metabolites are then excreted back into the small intestines via bile, where they can be eliminated in the feces or reabsorbed and taken back to the liver ("enterohepatic cycling"). Others enter the systemic circulation. Blood flow from the intestines to the liver is indicated by the red arrow. Blood flow from the liver to the systemic circulation is indicated by the blue arrow. Enterohepatic cycling is indicated by the light green arrow

ride used in industrial applications, to widely used drugs like acetaminophen (APAP), effectively target the liver.

Toxic liver injury is a challenge for clinicians, regulators, and public health practitioners. Clinically, it is difficult to diagnose it in patients, to determine the cause, and to predict its outcome. From a regulatory perspective, it is challenging to identify drugs with potential to cause hepatotoxicity during both preclinical and clinical development before they can reach the market. Finally, from a public health perspective, it is known that some chemicals in the environment can cause chronic liver disease and liver cancer, but it is difficult to determine the significance of those exposures and to monitor them in the real world. One possible approach to address these and other challenges is the development of biomarkers of exposure, diagnosis, prediction, and prognosis in toxic liver damage. Indeed, many investigators have focused their efforts in this area of research over the last 10 years. In this chapter, we will briefly review conventional liver injury biomarkers, discuss the challenges of diagnosis and prediction in toxic liver injury in more detail, and discuss the state-of-the-art liver injury biomarker research.

Current Liver Injury Biomarkers

Although the term "liver function tests" commonly refers to all of the conventional liver-centered biomarkers measured in serum or plasma, it is more accurate to divide them into separate categories: markers of (1) injury, (2) function, (3) proliferation, and (4) infection (Table 1). The major markers of liver injury are the

Table 1 Current liver injury biomarkers

Category	Biomarker	Mechanism of release/elevation/decrease
Biomarkers of injury	Alanine aminotransferase (ALT)	Release: cell death, membrane blebbing, increased expression (?)
	Aspartate aminotransferase (AST)	Release: cell death, membrane blebbing, increased expression (?)
	Alkaline phosphatase (ALP)	Release: cell death, increased expression (?)
	γ-Glutamyl transferase (GGT)	Release: increased expression, cell death
	Lactate dehydrogenase (LDH)	Release: cell death (?)
Biomarkers of function	Total and/or direct bilirubin	Elevation: impaired biliary excretion
	Prothrombin time (PT) or international normalized ratio (INR)	Elevation: impaired synthesis of coagulation factors
	Albumin and other serum proteins	Decrease: impaired synthesis
Biomarkers of proliferation	Alpha-fetoprotein (AFP)	Decrease: impaired proliferation and synthesis
Biomarkers of infection	Viral antigens and antibodies	Infection

aminotransferases, alanine aminotransferase (ALT), and aspartate aminotransferase (AST). Elevations in serum ALT and AST in patients with hepatitis were first identified by Arthur Karmen and Fernando De Ritis independently ca. 1955 (De Ritis et al. 1955; Karmen et al. 1955). It is generally thought that these enzymes are passively released from damaged or dying cells due to loss of plasma membrane integrity. The latter is supported by the observation that serum ALT values remain normal or relatively low during early TNF signaling-mediated apoptotic liver injury in mice – a carefully controlled process of cell implosion – but later increase with progression to secondary necrosis (Lawson et al. 1998; Leist et al. 1995). Additional mechanisms have also been proposed, such as membrane blebbing in which protrusions off the plasma membrane grow and burst, and increased expression (McGill 2016). The most common methods to measure ALT and AST today use a coupled enzyme reaction in which their pyruvate or oxaloacetate products, respectively, are further metabolized by lactate dehydrogenase or malate dehydrogenase, consuming NADH in the process. The loss of NADH in the reaction is then measured by absorbance (Karmen et al. 1955; McGill 2016). Other markers in this group include serum alkaline phosphatase (ALP) and γ-glutamyl transferase (GGT), though it should be noted that elevated ALP and GGT activity in circulation in liver disease may be due in part to induction of hepatic expression instead of or in addition to passive release due to injury (Pike et al. 2013; Teschke et al. 1977; Wu et al. 1976). In fact, this is widely accepted in the case of GGT due to reports in the 1970s that serum values for GGT correlate well with hepatic levels (Teschke et al. 1977). However, it should be noted that not all studies have been able to reproduce those findings and there is still some disagreement (Selinger et al. 1982).

The major markers of liver function are bilirubin and prothrombin time (PT). Bilirubin is a product of erythrocyte degradation (Erlinger et al. 2014). Aging erythrocytes are phagocytosed by macrophages, where the heme group of hemoglobin dissociates as a result of low lysosomal pH. Heme is then converted to biliverdin by heme oxygenase, and the biliverdin is reduced to bilirubin via bilirubin reductase. Bilirubin can then circulate in the blood in a complex with serum albumin. At the liver, bilirubin is taken up by hepatocytes, where it is conjugated with glucuronic acid. Finally, the conjugated bilirubin is transported into bile and excreted in feces via the intestines. Elevations in serum conjugated bilirubin (also called "direct" bilirubin because it reacts quickly in commonly used bilirubin tests without addition of reaction accelerants that are necessary to measure hydrophobic free bilirubin) are often observed in obstructive liver diseases (e.g., gallstones) due to impaired excretion as a result of the obstruction (Dufour et al. 2000). These elevations are sometimes also seen in severe hepatocellular damage (Dufour et al. 2000). Apparently, even the severely damaged liver retains some capacity to take up and conjugate bilirubin but cannot excrete it properly, resulting in elevated serum values. The liver is also the site of synthesis of all but one of the major coagulation factors, including the critical components fibrinogen (factor I), prothrombin (factor II), and factors V and X that are essential for the common pathway of coagulation. Thus, liver damage leads to reduced coagulation factor synthesis and therefore increased PT. PT is measured by mixing citrated plasma with calcium and thromboplastin

(a mixture of phospholipids and tissue factor) and measuring the time required to form a clot. The international normalized ratio (INR), a normalized value calculated from PT, is also increased in severe liver damage.

Alpha-fetoprotein (AFP) is unique as it is the sole marker of hepatocyte proliferation in use. Serum AFP is commonly measured as a tumor marker to diagnose, monitor, and prognosticate in hepatocellular carcinoma and some other cancers (Lai et al. 2017; Mizejewski 2004). It is also a critical part of birth defect screening, as maternal serum AFP is one of the tests used in the triple and quad screens (Crandall 1981; Mizejewski 2004). Recent studies have also demonstrated that it has prognostic value as a marker of liver regeneration and recovery in acute liver failure (ALF) (Schiødt et al. 2006; Schmidt and Dalhoff 2005; Singh et al. 2019; Varshney et al. 2017). However, it is not yet widely used for that purpose due to limitations including the fact that differences between transplant-free survivors and non-survivors are not clear until late in the progression of injury.

Finally, the markers of infection consist primarily of viral hepatitis antigens and antibodies (Peeling et al. 2017). These include IgM anti-hepatitis A virus antibodies (anti-HAV), IgM hepatitis B core protein antibodies (anti-HBc) and hepatitis B antigens (e.g., HBsAg), and, finally, hepatitis C antibodies (anti-HCV). PCR tests to detect and quantify viral load are also helpful in some cases (Peeling et al. 2017).

Although the focus of the remainder of this chapter will be markers of injury, it is useful to keep in mind that these other biomarkers of liver function, hepatocyte proliferation, and infection can complement investigation of liver injury by allowing one to probe the causes and predict outcomes of injury. We will now cover major issues with current liver injury biomarkers and recent developments in novel markers.

Limitations of the Current Biomarkers

The current biomarkers of liver injury, ALT and AST, are useful for detection and diagnosis of liver injury once a patient is symptomatic. However, they suffer several limitations. First, ALT and AST are not etiology-specific and therefore cannot be used to diagnose the cause of liver injury, excepting the modest utility of the AST/ALT ratio in identification of alcohol-induced liver disease. Second, these tests have very poor prognostic utility. ALT and AST values do not correlate with outcome after acute liver injury (Christensen et al. 1984; Dufour et al. 2000; Karvellas et al. 2017; Kuroda et al. 2021; McGill et al. 2014a; Tygstrup and Ranek 1986) and only weakly correlate in chronic liver diseases (Dufour et al. 2000). In addition, there is evidence that ALT at presentation is a relatively poor predictor of later liver injury in patients who present early after an insult such as APAP overdose (Dear et al. 2018). And finally, ALT and AST lack specificity for liver damage. Both enzymes are present in other tissues, particularly muscle and kidney (LaDue and Wroblewski 1956), limiting specificity for the liver in general. In addition, there are numerous reports of minor to moderate nonprogressive serum ALT elevations due to certain drugs in the absence of other evidences of liver injury

(Harrill et al. 2012; Singhal et al. 2014; Watkins et al. 2006), demonstrating less-than-desirable specificity for damage. Over the last two decades, a number of novel serum biomarkers have been discovered and proposed by research laboratories to address these limitations, and in some cases, their clinical value is just now being realized. These biomarkers are summarized in Fig. 2 and discussed in detail in the following sections.

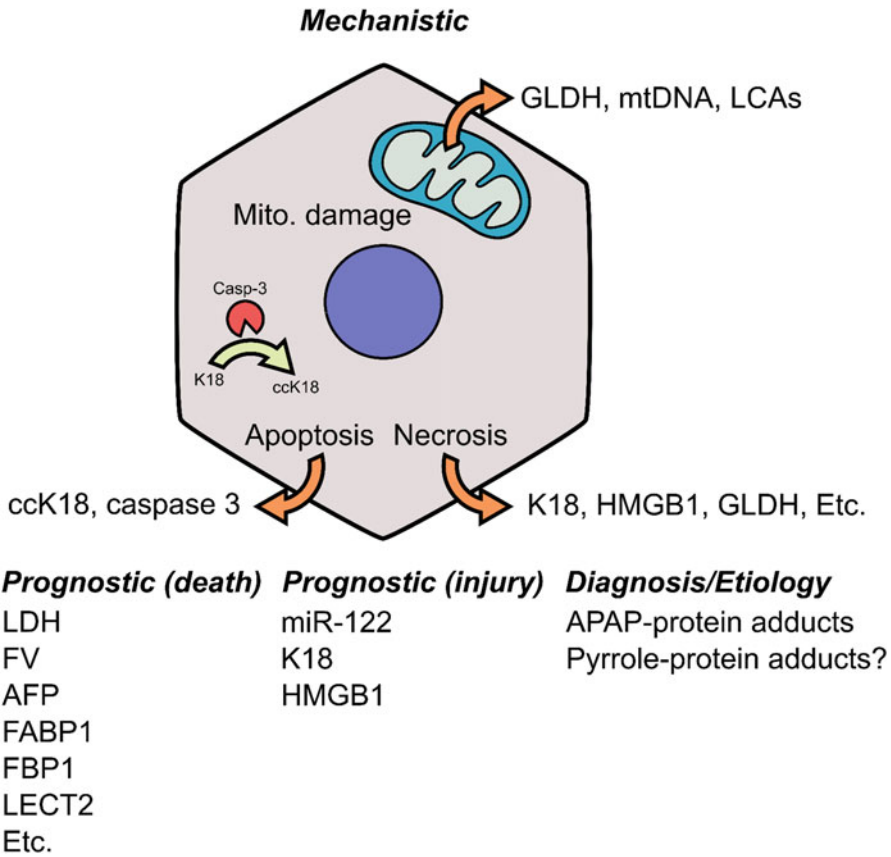

Fig. 2 Popular emerging biomarkers of liver injury. A number of biomarkers of liver injury have been developed with evidence to support various uses. Mechanistic/translational biomarkers indicate mitochondrial damage (glutamate dehydrogenase (GLDH), mitochondrial DNA (mtDNA), and long-chain acylcarnitines (LCAs)), apoptosis (caspase-cleaved keratin 18 (ccK18) and caspase-3 activity), and necrosis (full-length K18, total high = mobility group box 1 protein (HMGB1), and others). Some of these biomarkers also have prognostic utility, as indicated, in addition to lactate dehydrogenase (LDH), factor V (FV), alpha-fetoprotein (AFP), fatty acid-binding protein 1 (FABP1), fructose-1,6-bisphosphatase 1 [FBP1], leukocyte cell-derived chemotaxin 2 (LECT2), and others. Finally, emerging biomarkers for diagnosis/etiology include APAP-protein adducts for APAP overdose and pyrrole-protein adducts for pyrrolizidine alkaloids

Emerging Biomarkers of Etiology and Exposure

Currently, there is only one commonly used biomarker with sufficient specificity to diagnose the cause of toxic liver injury, aside from routine therapeutic drug monitoring to identify drug plasma concentrations outside normal ranges. We have known since the 1970s that the drug APAP is converted to a reactive metabolite that binds to proteins (Jollow et al. 1973) (Fig. 3). This fact has been exploited to develop APAP-protein adducts as a serum biomarker of APAP exposure and overdose. The earliest methods to measure APAP-protein adducts were immunoassays using antibodies against APAP or an APAP-cysteine conjugate (Roberts et al. 1987). Using this approach, APAP-protein adducts were initially measured in liver tissue and serum from APAP-treated mice and roughly a decade later in serum from APAP overdose patients (James et al. 2001; Pumford et al. 1989). Shortly after the first measurements in humans, an HPLC-based method was developed with electrochemical detection (Muldrew et al. 2002) followed later by mass spectrometry detection (Cook et al. 2015; McGill et al. 2011; Xie et al. 2015). Values $\geq$1 μM in the context of elevated ALT are considered specific for APAP overdose (Alonso et al. 2015; James et al. 2009; Khandelwal et al. 2011). Currently, only one Clinical Laboratory Improvement Ammendements (CLIA)-licensed laboratory offers serum APAP-protein adducts as part of their test menu (Acetaminophen Toxicity Diagnostics, LLC, in Little Rock, AR, United States), but expansion to other laboratories is possible in the coming years. In addition, the same company has developed a lateral flow immunoassay calibrated to the 1 μM cutoff (Roberts et al. 2017) and is currently seeking approval for the device from the US Food and Drug Administration. Thus, APAP-protein adduct testing for clinical use may become more common in the near future.

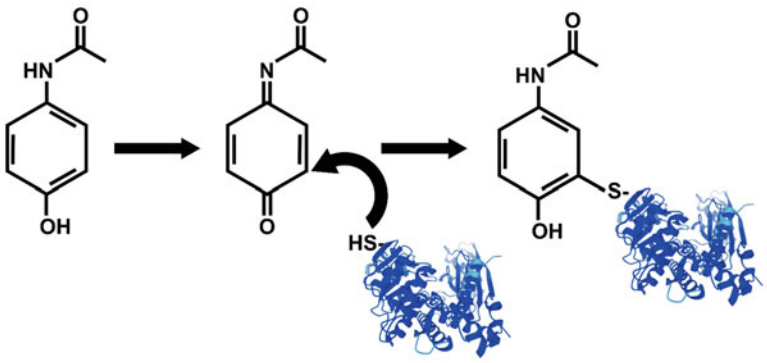

Fig. 3 Formation of APAP-protein adducts. Acetaminophen (APAP, left structure) is converted to N-acetyl-*p*-benzoquinone imine (NAPQI, middle structure), which has a partial positive charge on the meta carbon. The electrophilic carbon reacts with nucleophilic sulfhydryl groups (usually cysteine residues) on proteins (example protein shown in blue), reverting the structure of the drug back to APAP and forming the APAP-protein adduct (right structure)

For reasons described elsewhere, it will be a challenge to develop other biomarkers to determine etiology in the context of conventional drug-induced liver injury (DILI) (McGill and Jaeschke 2018, 2019). However, biomarkers of exposure may be useful in some cases of hepatotoxicity due to environmental chemicals. For example, there is growing interest in the measurement of pyrrole-protein adducts. Pyrrolizidine alkaloids are structural derivatives of pyrrolizidine produced by numerous plant species. Hundreds of these alkaloids have been identified in the wild and have long been recognized as a challenge in production of grazing livestock (Prakash et al. 1999). In recent years, concern regarding entry into the human food supply has intensified with recognition that these alkaloids may be a more common cause of human cancer than previously realized (He et al. 2021). As a result, a number of laboratories have developed and tested analytical methods to measure pyrrole-protein adducts (He et al. 2021; Gao et al. 2012; Ma et al. 2019, 2021; Ruan et al. 2015). Interestingly, these adducts may one day find clinical application as well, as pyrrolizidine alkaloids may be a cause of hepatotoxicity in some cases of herb-induced liver injury (Ruan et al. 2015).

Emerging Predictive Biomarkers

It is frequently suggested that it may be possible for clinicians to predict if a patient is likely to develop hepatotoxicity before writing a prescription for a DILI-associated drug for them based on a genetic marker or some other types of biomarker. This would not be an ideal approach due to the vanishingly low incidence of DILI among users of most DILI-causing drugs resulting in exceedingly low positive predictive values for most potential biomarkers (especially most genetic associations) (McGill and Jaeschke 2018, 2019; Stephens et al. 2021). One prominent exception is the association between human leukocyte antigen (HLA) B*5701 and abacavir hepatotoxicity (Mallal et al. 2002), which is useful due in part to the fact that abacavir causes other idiosyncratic reactions in addition to DILI leading to high overall incidence of adverse effects. Nevertheless, these genetic markers may be useful as a way to retrospectively identify a drug as a likely cause of hepatotoxicity after the fact. Thus, such biomarkers may have some real-world utility. A number of genetic associations with DILI are known, and many more continue to be identified. The affected genes encode products ranging from HLAs, to drug-metabolizing enzymes, to drug transporters (Stephens et al. 2021; Urban et al. 2014). At this point, however, few have clear potential for application in the near future.

Emerging Prognostic Biomarkers

There is an urgent need for improved biomarkers of prognosis in toxic liver injury in order to guide liver transplantation. Currently, although *N*-acetyl-*l*-cysteine is an effective treatment for APAP-induced liver injury when administered early after APAP overdose, few other specific treatments are available for toxic liver damage.

The major lifesaving treatment for liver injury patients who progress to liver failure is a liver transplant. However, donated liver are in limited supply, and those who do receive a transplant face serious postoperative challenges, including the possibility of graft rejection and development of infection due to the immunosuppressant drugs often required to stave off rejection. Better biomarkers could make the clinician's job easier when identifying which patients need a new liver to survive. To that end, a number of recent studies of varying quality have evaluated the prognostic potential of novel liver biomarkers over the past two decades, mostly using samples from APAP overdose patients because they are more widely available than samples from patients with other forms of toxic liver injury. A veritable alphabet soup of potential biomarkers has been described. These include full-length and caspase-cleaved keratin 18 (K18 and ccK18) (Bechmann et al. 2010; Church et al. 2019; Craig et al. 2011), high-mobility group box 1 protein (HMGB1) (Basta et al. 2015; Craig et al. 2011), glutamate dehydrogenase (GLDH) (Church et al. 2019; McGill and Jaeschke 2014), fatty acid-binding protein 1 (FABP1) (Karvellas et al. 2017), miR-122 (Church et al. 2019), fructose-1,6-bisphosphatase 1 (FBP1) (Wang et al. 2017), osteopontin (OPN) (Church et al. 2019; Srungaram et al. 2015), and leukocyte cell-derived chemotaxin 2 (LECT2) (Slowik et al. 2019). One of the more exciting recent reports found that a liver-regeneration-associated microRNA signature can also predict poor outcomes (Tavabie et al. 2021). So far, however, none have emerged as clear contenders for real-world use. Clinical adoption of these biomarkers has likely been impeded by (1) lack of FDA-approved reagents for their measurement and (2) lack of motivation to seek FDA approval on the part of commercial partners due to the relatively small market that exists for acute liver injury and liver failure patients. These issues may be circumvented by identification of biomarkers that already have approved reagents and are commonly measured in patients with other conditions. Two such "recycled" biomarkers that fulfill that criterion and have recently been shown to have prognostic value in acute liver injury are coagulation factor V (FV) and LDH. On one hand, admission FV values seem to correlate with positive outcomes (Patidar et al. 2021), while early LDH values seem to predict death (Vazquez et al. 2022). Nevertheless, further validation of both biomarkers is required.

In addition to biomarkers to predict death and therefore transplant need in severe injury, biomarkers have been tested to predict the development of later liver injury in patients who present early after a hepatotoxic exposure – before a rise in ALT. Among these, the most promising appear to be microRNA-122 (miR-122), K18, and HMGB1, with perhaps strongest performance from miR-122. All three displayed specificity >80% at 95% sensitivity to predict elevated peak ALT values (>100 U/L) in a validation cohort of APAP overdose patients who presented with ALT values in the normal range (Dear et al. 2018). In addition, miR-122 was shown to increase after moderate alcohol consumption with no change in ALT (McCrae et al. 2016), and although the data were preliminary in nature, miR-122 and K18 appeared to increase somewhat prior to ALT in two patients with hepatotoxicity caused by antitubercular drugs (Rupprechter et al. 2021). Finally, a recent study found that

GLDH and K18 were significantly elevated in serum from subjects with compensated cirrhosis compared to non-cirrhotic volunteers, while there was no difference in ALT between groups (McGill et al. 2021). Based on the prevalence of liver injury among early presenters after APAP overdose and the sensitivity and specificity achieved with these biomarkers (particularly miR-122), it seems likely that these markers are already approaching the limits of what is possible in this respect (McGill and Jaeschke 2018, 2019), and the pursuit of more widespread clinical adoption of one or more of these markers to predict injury in early-presenting APAP-induced liver injury patients could be appropriate at this time.

Emerging Biomarkers with Greater Specificity

There are two major challenges with the use of ALT and/or AST to detect and monitor liver injury during clinical trials, and both could be considered issues of specificity. First, these aminotransferases have poor utility to discriminate between liver and muscle damage in clinical trials involving patients with musculoskeletal diseases (Schomaker et al. 2020). Both ALT and AST are highly expressed in muscle and kidney tissue in addition to hepatocytes (LaDue and Wroblewski 1956), so both increase in the context of muscle damage. Currently, the combination of creatine kinase (CK) and ALT may be used to explore the source of the aminotransferases. For example, if a patient in a clinical trial has minor to modest ALT elevations with extremely high CK values and no major risk factors for liver damage, then one may assume that the ALT is elevated secondary to muscle injury. On the other hand, if ALT is much greater than CK, then the liver is a likely source of the ALT. However, a better approach would be to compare with a biomarker that is almost solely expressed in the liver. It is thought that GLDH is highly localized to mitochondria in the liver or at least that it is much more abundant there than in most other tissues (Schmidt and Schmidt 1988). Indeed, recent evidence has demonstrated that GLDH has clear utility to help differentiate liver and muscle damage (Schomaker et al. 2020). To that end, the GLDH subsection of the Hepatotoxicity Working Group of the Critical Path Institute (a public-private collaboration between the US FDA, pharmaceutical companies, and academic researchers) is currently working toward qualification of GLDH for use in clinical trials.

In addition to tissue specificity, there is the issue of specificity for injury. The US FDA recognizes that modest ALT elevations frequently do not indicate clinically significant, progressive liver damage (FDA 2009). Some drugs are known to cause transient ALT elevations in a significant proportion of patients who take them without leading to a single case of serious injury, liver failure, or death (Gracon et al. 1998; Harrill et al. 2012; Singhal et al. 2014; Watkins et al. 1994). A recent study using an unbiased, untargeted proteomics approach to compare serum between a model of benign ALT elevations and toxic ALT elevations revealed a number of potential biomarkers with greater specificity for injury, which were then confirmed to be elevated in serum from patients with APAP

hepatotoxicity (Vazquez et al. 2020). Chief among the candidate biomarkers was alcohol dehydrogenase 1A1 (ALDH1A1) and aldehyde dehydrogenase 2 (ADH2) (Vazquez et al. 2020). The authors of that manuscript propose a screen-and-confirm algorithmic approach in which ALT is used to screen for liver injury during clinical trials, and one of the candidate biomarkers is used to confirm it (Vazquez and McGill 2021). There is still much more work to be done to validate these novel injury-confirmation markers, but it appears to be a promising future direction based on the available data.

Mechanistic Biomarkers

Another potential use of novel biomarkers is investigation of liver injury mechanisms. A "mechanistic" biomarker is one that depends upon and therefore provides insight into a process that drives the pathophysiology of a disease at a fundamental level (i.e., molecular, cellular, or tissue). The term frequently refers to biomarkers intended for use as a way to monitor response to cancer treatments with specific therapeutic actions (De Haas et al. 2008; Lopez-Girona et al. 2011; Keen et al. 2014; Sorensen et al. 2009; Ueno et al. 2005) but has been applied to other contexts including liver injury in recent years (McGill and Jaeschke 2014). Several promising mechanistic biomarkers have been identified in patients with liver injury (Fig. 2). McGill et al. demonstrated that elevated serum levels of GLDH and mitochondrial DNA (mtDNA) in APAP hepatotoxicity likely reflect mitochondrial damage (McGill et al. 2012; McGill and Jaeschke 2021). In addition, nuclear DNA fragments in serum could reflect release of mitochondrial intermembrane endonucleases as a result of mitochondrial dysfunction (McGill and Jaeschke 2021). Similar data were reported for serum long-chain acylcarnitines (Bhattacharyya et al. 2014; McGill et al. 2014b), which are normally metabolized in mitochondria and therefore accumulate when mitochondria are damaged. On the other hand, ccK18 and the ratio of ccK18 to full-length K18 are markers of caspase-dependent apoptosis (Caulín et al. 1997; Leers et al. 1999) that are elevated in serum from some patients with toxic ALF (Craig et al. 2011; Woolbright et al. 2017). Direct measurement of caspase activity in serum also appears to be a useful measure of apoptosis in liver injury (McGill et al. 2012), while total HMGB1 may more commonly represent necrosis (McGill and Jaeschke 2014). Finally, a number of cytokines increase in serum during toxic liver damage and likely reflect inflammation that may affect injury or recovery (McGill and Jaeschke 2014). A new direction in mechanistic biomarkers in liver disease is those that reflect liver regeneration and therefore may be useful for prognosis as well. Two examples are Lect2 (Slowik et al. 2019), which is involved in inflammation, and phosphatidic acid, which appears to promote liver regeneration by inhibiting glycogen synthase kinase 3β (Clemens et al. 2019; Lutkewitte et al. 2018). The latter has been shown to increase in liver tissue and serum from mice with APAP hepatotoxicity and in serum from humans with APAP-induced liver injury, but its prognostic value remains to be determined.

Summary and Conclusions

Recent years have brought the discovery and preliminary evaluation of numerous novel biomarkers of liver injury. Measurement of serum APAP-protein adducts has clear value to diagnose APAP-induced liver injury and is already being measured clinically in some parts of the United States. Other biomarkers (factor V, LDH, alpha-fetoprotein, FABP1, FBP1, etc.) hold promise for prediction of death in severe liver injury but require further validation, while others (miR-122 and K18) may predict later liver injury in early presenters after APAP overdose. Finally, some (K18 and ccK18, GLDH, mtDNA, long-chain acylcarnitines, caspase activity, and regeneration markers) appear to have mechanistic value for translational research. Future work should focus on validating more of these biomarkers for clinical use. In addition, identification of more and potentially better biomarkers may be achieved through the use of novel tools, such as artificial intelligence approaches (Umbaugh and Jaeschke 2021).

Applications to Prognosis

In this chapter, we reviewed a selection of biomarkers that appear to predict (1) later injury in early-presenting patients with acetaminophen-induced hepatotoxicity (e.g., microRNA-122) (Dear et al. 2018) and (2) poor outcomes in severe toxic liver injury and/or acute liver failure (e.g., alpha-fetoprotein, osteopontin, factor V, lactate dehydrogenase) (Schmidt and Dalhoff 2005; Church et al. 2019; Patidar et al. 2021; Vazquez et al. 2022). MicroRNA-122, in particular, appears to be approaching the maximum predictive value for the former. The latter biomarkers require further validation in larger studies.

Applications to Other Diseases or Conditions

The focus of this chapter was on biomarkers of toxic liver injury. However, many of the biomarkers presented here are likely elevated and have clinical value in other forms of liver disease. Indeed, a few of these biomarkers are known to be elevated in fatty liver disease (Lee et al. 2020), cirrhosis (McGill et al. 2021), and other chronic hepatic diseases. They may also be useful in acute-on-chronic liver failure.

Mini-dictionary of Terms

- **Acute liver failure:** A condition in which liver function is rapidly compromised as a result of liver injury, leading to coagulopathy and encephalopathy within a short time frame and without evidence of prior liver disease
- **Drug-induced liver injury:** Liver injury caused by drugs that may present with a dose-response pattern characteristic of either intrinsic or idiosyncratic hepatotoxicity

- **Etiology:** The original cause of a disease or condition
- **Mechanistic biomarker:** A biomarker that provides some kind of insight into the mechanism(s) of disease
- **Positive predictive value:** The percentage of patients with a positive biomarker result who actually have the condition of interest
- **Predictive biomarker:** A biomarker that can predict the onset of an illness or condition before the illness or condition has developed
- **Prognostic biomarker:** A biomarker that can predict the outcome of an illness or condition after the illness or condition has developed
- **Sensitivity:** The percentage of patients with a condition that have a positive biomarker result
- **Specificity:** The percentage of patients without a condition that have a negative biomarker results

Key Facts of Acetaminophen

Acetaminophen was first synthesized and accidentally discovered to be an effective fever reducer in the late 1800s.

Due to unwarranted concerns that it can cause methemoglobinemia, it was not widely available to consumers until the 1950s.

Acetaminophen is now the most commonly used drug in the United States.

The first reports of acetaminophen-induced toxic liver injury appeared in the 1970s.

Today, acetaminophen is the single most commonly implicated cause of acute liver failure in the United States, the United Kingdom, and several other countries.

Key Facts of Acute Liver Injury

Acute liver injury is the sudden onset of severe liver damage.

Circulating alanine aminotransferase values >300 U/L are highly specific for acute liver injury, though they do not provide any insight into the etiology and have little prognostic value.

The most common causes in the United States are hypoxic hepatitis, drug-induced liver injury (especially acetaminophen hepatotoxicity), and pancreatobiliary diseases.

Outcomes are generally good unless the patient progresses to acute liver failure.

Outcomes are generally better for hypoxic hepatitis than for other causes, such as drug-induced liver injury.

Key Facts of Acute Liver Failure

Acute liver failure is defined as coagulopathy and encephalopathy developing within days to weeks of acute liver injury in the absence of prior chronic liver disease.

Despite recent progress toward improved outcomes, acute liver failure remains highly fatal with overall mortality around 25–30%.

Drug-induced liver injury is the single most common cause of acute liver failure and related deaths in most countries.

Acetaminophen is the single most commonly implicated agent in toxic acute liver failure.

Key Facts of Drug-Induced Liver Injury

Drug-induced liver injury is one of the most common causes of acute liver injury and acute liver failure in the United States and several other countries.

There are two forms: intrinsic and idiosyncratic.

Intrinsic drug-induced liver injury is characterized by a clear dose-response, with high predictability, meaning that all or nearly all individuals who consume a dose greater than some threshold will experience liver damage.

Idiosyncratic drug-induced liver injury is challenging to predict because it occurs in only a small proportion of individuals exposed to commonly used pharmacologic doses, and most cases appear to involve an immune system component.

Altogether, drug-induced liver injury is by far the most common cause of acute liver failure and one of the most common causes of acute liver injury.

Key Facts of Pyrrolizidine Alkaloids

Pyrrolizidine alkaloids are a class of naturally occurring toxins present in many plants of agricultural significance.

These compounds are known to cause acute liver damage in livestock and humans and are likely also carcinogenic.

Recent data indicate that dietary exposure to these compounds may be more common than previously thought, with potential clinical significance.

Men may be more sensitive to their toxic effects than women.

Some insects feed on plants that produce pyrrolizidine alkaloids, accumulate the compounds within their own tissues, and use them as either a poisonous deterrent or as a precursor for pheromone synthesis.

Summary Points

- The liver is more susceptible to toxic damage than most other organs due to its unique anatomy and physiology.
- Current liver injury biomarkers can be grouped into markers of injury, function, infection, and proliferation.
- Current liver injury biomarkers are nonspecific and lack prognostic value either because their values do not correlate with outcomes or because they increase too late in the disease to be useful.
- Numerous studies over the last two decades have identified biomarkers that may be useful for determination of etiology in patients with liver injury, prediction of

drug-induced liver injury in patients before they begin taking a drug, diagnosis or determination of prognosis once injury occurs, and exploration of injury mechanisms.

- Low incidence or prevalence of idiosyncratic drug-induced liver injury is a major challenge in identification of biomarkers for diagnosis and prediction, but biomarkers for prognosis are ripe for further exploration and development.

Cross-References

► Biomarkers of Alcohol Toxicity
► Drug-Induced Nephrotoxicity and Use of Biomarkers

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Biomarkers for Assessing Mycotoxin Exposure and Health Effects

13

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Abstract

A variety of adverse health effects can be determined after exposure to mycotoxins, the toxic metabolites produced by some fungi species during growth on food products. Considering the health risks posed by mycotoxins, regulatory agencies throughout the world have adopted regulations for these compounds in susceptible foods, to prevent their dietary exposure in humans. The estimation of real exposure to mycotoxins through food consumption is a difficult task, although recent advances provided reliable measures through biomarkers found in biological fluids. A biomarker is generally estimated by a biochemical or physiological change occurring within an organism based on the extent of exposure that can be linked to a possible health disorder or disease. In this chapter, a comprehensive description of toxicokinetic basis for exposure and disease biomarkers of main mycotoxins is presented, along with a discussion on the adverse health effects of these toxic compounds in human populations.

Keywords

Aflatoxins · Ochratoxin A · Fumonisins · Trichothecenes · Zearalenone · Human biomonitoring

Abbreviations

AFB_1	Aflatoxin B_1
AFB_1-FAPY	Aflatoxin B_1- formamidopyrimidine
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
AF-N^7-Gua	Aflatoxin-N^7-guanine
AFs	Aflatoxin(s)
ALP	Alkaline Phosphatase
AST	Aspartate aminotransferase
BUN	Blood urea nitrogen
CI	Confidence interval
DNA	Deoxyribonucleic acid
DOM-1	Deepoxideoxynivalenol
DON 15-GlcA	Deoxynivalenol 15-glucuronide
DON 3-GlcA	Deoxynivalenol 3-glucuronide
DON	Deoxynivalenol
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FBs	Fumonisin(s)
GPx	Glutathione peroxidases
Gua	Guanine
HCC	Hepatocellular carcinoma

HCEC-1CT	Human Colonic Epithelial Cells
HK-2	Human kidney tubular epithelial cells
HSA	Human serum albumin
IARC	International Agency for Research on Cancer
IGF1	Insulin-like Growth Factor
JECFA	Joint FAO/WHO Expert Committee on Food Additives
mRNA	messenger RNA
OTA	Ochratoxin A
PPE	Porcine pulmonary edema
ppm	Parts per million
Sa	Sphinganine
So	Sphingosine
SOCS3	Suppressors of cytokine signaling
SOD	Superoxide dismutase
TP	Total Protein
ZEN	Zearalenone
α-ZAL	α -zearalanol
α-ZEL	α -zearalenol
ββ-ZAL	ββ-zearalanol
ββ-ZEL	ββ-zearalenol

Introduction

Mycotoxins are toxic compounds produced as natural secondary metabolites by certain species of fungi in a variety of agricultural commodities, widely affecting commodities consumed by humans and animals, from field to storage. The main species of mycotoxin-producing fungi are the genera *Aspergillus, Fusarium,* and *Penicillium* (Oliveira et al. 2014). These species are often found in the agricultural environment, which represents a problem for the worker's health, owing to high occupational exposure through inhalation or cutaneous absorption of mycotoxins produced and sedimented in airborne particles.

More than four hundred types of mycotoxins have been reported across the world; however, the most frequently detected and regulated are the aflatoxins (AFs), ochratoxin A (OTA), zearalenone (ZEN), fumonisins (FBs), and deoxynivalenol (DON). Fungi of the *Aspergillus* species are the major producers of mycotoxins, producing mainly AFs through *A. flavus*, *A. parasiticus,* and *A. nomius*, OTA through *A. ochraceus*, *A. niger,* and *A. carbonarius,* and FBs through *A. niger*. OTA mycotoxin is also produced by *Penicillium* verrucosum (Sweeney and Dobson 1998; Richard 2007; Köszegi and Poór 2016). Furthermore, *Aspergillus sp*. produces the mycotoxins with the greatest carcinogenic potential (IARC 2002). Regarding *Fusarium* sp., the main producers are *F. verticillioides* and *F. proliferatum* producing FBs, *F. culmorum* producing DON, and *F. graminearum* producing ZEN and DON (Frisvad et al. 2011; Ostry et al. 2017).

As mycotoxins can cause a variety of adverse health effects, such as immune deficiency and cancer, for mainly causing damage to organs and central nervous system, and thus posing a serious risk to human health, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) was created to take responsibility by assessing food safety for mycotoxins. Therefore, through the assessments performed by JECFA, the Codex Alimentarius Commission determines international standards to limit exposure to mycotoxins in certain foods. With the defined standards, each country/continent establishes its legislation.

Regulations for Mycotoxins

The occurrence of mycotoxins is a concern that occurs in all continents, but because it is affected by climatic conditions, exposure to mycotoxins varies between geographic areas (Paterson and Lima 2011). Thus, regulations and guidelines change according to the mycotoxin incidence rates for each region. Table 1 provides a summary of human food consumption regulations regarding mycotoxins worldwide. In the European Union (EU), mycotoxin regulations for foods cover a range of foods, with the maximum permitted levels of AFs for susceptible foods, such as peanuts, almonds, cereals, infant formulae, and milk, ranging from 0.025 to 15 μg/kg. The maximum level allowed by the EU for DON ranges from 200 to 1750 μg/kg, for ZEN from 20 to 350 μg/kg, for FBs from 200 to 4000 μg/kg, and for OTA from 0.5 to 80 μg/kg. In the USA, the AFs regulations cover for all foods, except milk, a maximum level at 20 μg/kg, and for aflatoxin M_1 (AFM_1) in milk a maximum at 0.5 μg/kg. USA regulations also have guidance level for DON in finished wheat products (1000 μg/kg) and for FBs in corn and corn products (2000 to 4000 μg/kg). Brazil, China, and Korea have regulations for AFs, ZEN, OTA, FBs, and DON for various grain-based foods. Japan has only maximum level for AFs in all food (10 μg/kg) and milk (0.5 μg/kg) and guidance level for DON in wheat and wheat products at 1100 μg/kg. Indonesia presents maximum level for AFs in corn, nuts, spices, and milk and derivatives, and for DON, FBs and OTA for same cereals, as in Malaysia which has legislation for AFs and OTA in cereals.

Importantly, the primary intention of legislative guidance is to regulate and provide legal basis for inspection of foods susceptible to contamination existing in their territories, thus reducing the human exposure to mycotoxins. This exposure is measured more efficiently through biomarkers, which is discussed in detail below.

Biomarkers of Exposure to Mycotoxins

Biomarkers in human biological fluids indicate the presence of a chemical agent resulting from some exposure, being able to quantify the magnitude of this contact. Therefore, the use of urinary and serum biomarkers was introduced in the estimation of mycotoxins exposure considering the correlation with the internal dose of the original compounds acquired through the ingestion of contaminated food, or through

Table 1 Summary of worldwide regulations regarding mycotoxins in foods

Location	Commodity	Mycotoxin	Maximum level or guidance range (μg/kg)	Reference
European Union	Groundnuts (peanuts), almonds, pistachios, apricot kernels, tree nuts, dried fruit, hazelnuts, and Brazil nuts to be subjected to sorting, or other physical treatment, before human consumption	AFs	15.0	European Union (2006)
	Groundnuts (peanuts), almonds, pistachios, apricot kernels, hazelnuts, Brazil nuts, and spices for direct human consumption and tree nuts and dried fruit, rice, and maize to be subjected to sorting, or other physical treatment	AFs	10.0	
	Raw milk	AFM_1	0.05	
	Processed cereal-based foods and baby foods for infants and young children	AFB_1	0.1	
	Infant formulae and follow-on formulae, including infant milk and follow-on milk	AFM_1	0.025	
	Unprocessed cereals (excluding durum wheat, oats, and maize)	DON	1250 to 1750	
	Cereal flour, maize-based products, wheat-based products, and processed cereal-based baby foods	DON	200 to 750	
	Unprocessed cereals and derivatives, dried wine fruit, wine coffee and derivatives, spices and derivatives, baby foods, wheat gluten, and grape juice	OTA	0.5 to 20	
	Unprocessed maize, maize and maize-based products	FBs	200 to 4000	
	Unprocessed cereals, cereals for direct consumption, maize-based products, and wheat-based products	ZEN	20 to 400	
USA	All foods except milk	AFs	20	Food & Drug Administration (2000, 2001, 2010)
	Milk	AFM_1	0.5	
	Wheat-based products	DON	5 to 10	
	Corn-based products	FBs	2000 to 4000	

(continued)

Table 1 (continued)

Location	Commodity	Mycotoxin	Maximum level or guidance range (μg/kg)	Reference
Brazil	Milk and milk derivatives, cereals, Brazil nuts, nuts, dried fruit, peanuts, species, beans, almonds, cocoa, and derivatives	AFs	0.5 to 20	Agência nacional de vigilância sanitária (2014)
	Cereals, coffee, wine and derivatives, dried fruit, beans, almonds, cocoa, and derivatives	OTA	2 to 750	
	Rice and derivatives, cereal-based foods for children's nutrition, wheat and wheat-based products, maize, and malted barley	DON	200 to 3000	
	Popcorn, maize-based foods for children's nutrition, and maize-based products	FBs	200 to 2500	
	Maize and wheat to be subjected to treatment, rice and derivatives, maize-based products, and wheat-based products	ZEN	20 to 800	
China	Grain and grain products, beans and products, nuts, seeds, oils and products, condiments, infant formula, milk, and milk products	AFs	0.5 to 20	Global Agricultural Information Network (2018)
	Grain and grain products, beans and products, alcoholic beverage, nuts, seeds, and coffee	OTA	5 to 10	
	Grain and grain products	DON	1000	
	Grain and grain products	ZEN	60	
Korea	Grains, beans, peanut, nuts, processed cereal products, spices, wheat flour, popcorn, condiments, rice, milk, and foods for infants	AFs	0.5 to 15	Romer Labs (2021)
	Grain and grain products, coffee, pepper, grape juice, and foods for infants	OTA	0.5 to 20	
	Grain and grain products, corn and corn-based products, cereals, and foods for infants	DON	0.2 to 2000	
	Grain and grain products, popcorn, maize, and maize-based products	FBs	1000 to 4000	
		ZEN	20 to 200	

(continued)

Table 1 (continued)

Location	Commodity	Mycotoxin	Maximum level or guidance range (μg/kg)	Reference
	Grain and grain products and foods for infants			
Japan	All food and milk	AFs	0.5 to 10	Romer Labs (2021)
	Wheat and wheat products	DON	1100	
Malaysia	Groundnuts, almonds, hazelnuts, pistachios, Brazil nuts, cereal-based food for infants and children, milk, and infant formula	AFs	0.025 to 15	Romer Labs (2021)
	Cereal-based food for infants and coffee	OTA	0.5 to 10	
Indonesia	Corn, nuts, spices, and milk	AFs	0.5 to 20	Romer Labs (2021)
	Cereal and cereal products, coffee, spices, and beer	OTA	0.5 to 20	
	Wheat, wheat-based products, maize, and maize-based products	DON	200 to 1750	
	Maize and maize-based products	FBs	1000 to 2000	

AFs: total aflatoxin; FBs: total fumonisin; DON: deoxynivalenol; OTA: ochratoxin A; ZEN: zearalenone

the inhalation of contaminated airborne particles. Barely for mycotoxins with known toxicokinetic, it is possible to quantify the biomarkers generated by the interaction with the human organism.

Biomarkers of Aflatoxin Exposure

Concerning AFs, AFB_1 is the most toxic compound with carcinogenic potential in humans (IARC 2002). After ingestion, AFB_1 is biotransformed by the liver through the cytochrome P450 system, generating hydroxylated metabolites, such as aflatoxins M_1, Q_1, P_1, and B_{2a}. The hydroxylated compounds are excreted in the urine and milk of animals such as dairy cattle and lactating women, especially AFM_1 also excreted in urine. In the liver, AFB_1 may also undergo epoxidation, forming the AFB-8,9-epoxide responsible for the covalent bonds with nucleic acids (DNA) and with serum albumin, thus generating the AFB_1-N^7-guanine and AFB_1-lysine adducts, respectively. AFB_1-N^7-guanine adduct or AFM_1 in urine is considered as a biomarker of short-term exposure to AFs, whereas measurement of the AFB_1-lysine adduct in human serum indicates a long-term exposure (Jager et al. 2016). Groopman et al. (1992a) concluded that the percentages of AF metabolites excreted

in urine in relation to ingested are 4.4% in women and 7.6% in men. For AFM_1, the percentage excreted in the urine ranged from 1.23 to 2.18% for men and from 1.30 to 1.78% for women, of the total AFB_1 consumed (Zhu et al. 1987).

Biomarkers of Ochratoxin A Exposure

About 40 to 60% of the ingested OTA is passively absorbed from the gastrointestinal tract, favoring the binding to plasma proteins upon achieving the bloodstream. The affinity and degree of binding of OTA to these proteins is responsible for the variations observed in the half-life of the compound between the animal species already studied, reaching up to 99% in humans. Thus, the plasma half-life in humans remains an average of 35 days, being a long-exposure biomarker (Hagelberg et al. 1989; EFSA 2006; Ringot et al. 2006). Importantly, OTA is not removed by glomerular filtration, but slowly eliminated in the urine. OTA is transported through the system and distributed to different tissues and organs, mainly in kidneys, muscles, and liver. In the liver, OTA is biotransformed by cytochrome P450 enzymes generating hydroxyochratoxin A metabolites, mainly ochratoxin alpha (OTα), a less toxic compound than OTA. Schlatter et al. (1996) concluded that 50% of consumed OTA is excreted in 24-hour urine, but the relationship between OTA and OTα levels in urine samples remains unclear (Groves et al. 1998; Ringot et al. 2006; Muñoz et al. 2010).

Biomarkers of Fumonisin Exposure

Regarding FBs, there is evidence that FB_1 can be metabolized by the intestinal flora (Riley et al. 2011). Daud et al. (2019) concluded that the human fecal microbiota is capable of degrading FB_1, but the degradation products have not been fully identified. Studies with different animal species have shown that FB_1 toxin is poorly absorbed after oral ingestion and excreted in small amounts in the urine, being less than 2% (Shephard et al. 2007). FB_1 is quickly detected in the circulation blood and little accumulated in the liver and kidney, also being excreted in bile (Orsi et al. 2009). The half-life of FB1 is nearly 128 minutes for a 70 kg person, as estimated through extrapolation from toxicological studies in animals (Delongchamp and Young 2001). A study with human potential consumers of food-containing FBs showed that only 0.5% of ingested FB_1 is eliminated in 24-hour human urine (Riley et al. 2012).

Biomarkers of Deoxynivalenol Exposure

DON ingested with contaminated foods is rapidly absorbed in the upper gastrointestinal tract, where it undergoes de-epoxidation and generates the de-epoxi-

deoxynivalenol (DOM-1). DON is mainly eliminated by conjugation with glucuronic acid through human liver microsomes, forming the metabolites DON 15-glucuronide (DON 15-GlcA) and DON 3-glucuronide (DON 3-GlcA), in addition to glucuronidation products and DON isomeric. Following the urinary and fecal excretion, the human fecal microbiota can metabolize DON into DOM-1. Most metabolites are excreted through the urine 24 hours after exposure, with a relatively short half-life and a mean ratio of 50% and 72% of the total DON ingested by men and women, respectively (Vidal et al. 2018a).

Biomarkers of Zearalenone Exposure

Zearalenone, after being consumed orally, is metabolized by the intestine or liver by hydroxylation after considerable enterohepatic recirculation, mainly producing α- and ββ-zearalenol (α-ZEL and ββ-ZEL, respectively). These compounds also undergo an additional reduction and originate α- and ββ-zearalanol (α-ZAL and ββ-ZAL, respectively). ZEN can also generate metabolites conjugated to glucuronic acid, facilitating the presystemic elimination of toxins in the urine, but due to enterohepatic recirculation they have prolonged total body clearance (Kuiper-Goodman et al. 1987; Borchers et al. 2010). The formation of α-ZEL is considered a bioactivation reaction as it presents a higher toxicity than the original compound, and because they are produced by intestinal Caco-2 cells. The production of β-ZEL, on the other hand, indicates a detoxification process (Videmann et al. 2008). Based on exposure studies in humans and data obtained from biotransformation studies with pigs, a 24-hour urinary ZEN excretion of 36.8% of the total consumed was estimated for humans (Gambacorta et al. 2013). ZEN biomarkers can also be detected and studied in serum, as ZEN binds to human serum albumin thus transforming the parent compound into ZEN-HSA (Poór et al. 2017). Furthermore, in animals and human studies, ZEN has been shown to be rapidly absorbed after oral ingestion and that human intestinal microflora is not capable to degrade the ZEN.

Biomarkers of Mycotoxins Health Effects

Mycotoxins, when ingested in larger amounts or for longer time periods above the safety limits, can become a risk to animal and human's health. The most frequent mode of exposure to mycotoxins is through oral ingestion, although it may also occur through inhalation or dermal contact (Marin et al. 2013). The impact of exposure to mycotoxins on health may differ depending on the type of mycotoxin, levels of exposure, health status, and age of the individual exposed. The combined effect of various mycotoxins and other chemicals to which the individual is exposed to is also important in defining the extent of effect on health.

Human exposure toward various mycotoxins was used to be estimated through dietary surveys, prior to the introduction of biomarkers of exposure. This practice, however, was subjected to a recall prejudice. Randomly selected and investigated food materials most of the time do not reflect vast differences in mycotoxins concentrations that often happen in families with diverse backgrounds. In comparison, the biomarkers carry a potential to provide a dependable approximation of exposure through all sources in exposed populations and, even in few cases, may help to check an action mechanism (Riley et al. 2011).

The biomarkers have proven to be an effective tool in determining the extent of exposure to various chemicals and their effect in recent times. Biomarkers are measurable biochemical or molecular gages of exposure or the biological response to a mycotoxin which may be precisely related to ultimate health impact (Baldwin et al. 2011). The biomarkers for various mycotoxins specifically correlate with the quantity of that particular toxin ingested. The biomarkers may include protein or DNA adducts, metabolized forms of parent toxins, or other agents in urine or plasma/serum of exposed individual. A biomarker of effect is usually estimated by biochemical or physiological, behavioral, or other changes that occur inside an organism based on the extent of exposure which may be linked with a possible health disorder or ailment.

Aflatoxins

Aflatoxins are readily absorbed into the blood stream from the site of exposure. The two major routes of aflatoxin exposure include (i) the ingestion of either directly contaminated food or food containing carryover aflatoxins from feed of animals such as milk or milk products and various animal tissues, (ii) the inhalation of dust particles contaminated with AFB_1 from the food that occurs especially in food industries and factories (Agag 2004, Coulombe 1993). The aflatoxins, after being absorbed into the blood stream, are then transferred to body tissues and to the liver. The liver transforms the aflatoxin into a reactive epoxide intermediate or into a hydroxylated less toxic AFM_1 (Wild and Montesano 2009; Wu and Khlangwiset 2010). The epoxidation of AFB_1 to the exo-8, 9-epoxide by CYP450 enzymes is a critical step in the genotoxic pathway of aflatoxin.

Exposure biomarkers and biomarkers of toxic effect for aflatoxins have been validated in various studies in both animals and humans as presented in Table 2. The reactive epoxide form binds to DNA and the albumin in the serum (Wild and Montesano 2009, Wu and Khlangwiset 2010). The formation of AFB_1-DNA adducts by AFB_1 exo-8,9 epoxide results in functional changes of DNA (Raney et al. 1993). These adducts are formed as a result of reactive nature of epoxide and its affinity toward guanine bases in DNA causing the formation of aflatoxin-N^7-guanine (Gua) (Guengerich 2001). The dose-response association between both aflatoxin-N^7-Gua adduct and AFM_1 and the incidence of hepatocellular carcinoma was first studied in animals (Groopman et al. 1992b).

Table 2 Aflatoxin exposure, effected biological parameters, and health implications

Location	Type of sample / species	AFs exposure level	Number of samples	Effected biological parameters	Health implications	Reference
Kenya	Serum samples of children (1-14 years)	100% serum samples of children were positive with AFB_1 (geometric mean: 45.38 pg/mg albumin)	425	Increased level of low antihepatitis B antibodies for a unit increase in AFB_1, anti-HBs reduced by 0.91 mIU/ml (OR = 0.35; $p < 0.01$); downregulation of cytokines	Reduced effectiveness in hepatitis B infection particularly in malnourishment	Githanga et al. (2019)
Gambia	Serum samples of children (6-9 years)	93% of the children were positive for AF-alb adducts (geometric mean: 22.3 pg/mg)	427	Reduced levels of secretery immunoglobulins	Modification of immune system	Turner et al. (2003)
Taiwan	Zebrafish embryos and larvae	In vitro exposure of 6 h postfertilized embryos with AFB_1 at 15-75 ng/ml	Not given	Hyperlocomotion, reduced startle response, abnormal morphology of hindbrain neurons, and trigeminal ganglion downregulation of ngfa and atp1b1b genes expression and upregulation of prtga gene	Behavioral changes, alteration neuronal morphology, and gene expression	Wu et al. (2019)
USA	Primary human brain microvascular endothelial cells (HBMEC), human umbilical vein endothelial	AFB_1 exposure dose 3-32 nM	Not given	Death of primary HBMEC (85%), HUVEC (22%); DNA adduct formation in	Cytotoxicity of brain endothelial cells and more liver damage	Qureshi et al. (2015)

(continued)

Table 2 (continued)

Location	Type of sample / species	AFs exposure level	Number of samples	Effected biological parameters	Health implications	Reference
	cells (HUVEC), human hepatocellullar carcinoma cells (Huh7)			HBMEC, Huh7, and HUVEC		
USA	Primate cells (Cos-7 cells)	Oligodeoxynucleotides (12mer; 400 pmol) containing AFB_1-FAPY were inserted in DNAs	Not given	G-T substitutions in DNA, mutagenicity	Increased risk of hepatocellular carcinoma	Lin et al. (2014)

The biomarker AF-N^7-Gua can be a predictive of hepatocellular carcinoma induced by aflatoxins (Bbosa et al. 2013). Aflatoxin-N7-Gua in urine has been validated as biomarker of biologically effective dose (Groopman et al. 1992a; Kensler et al. 2011). After acid hydrolysis, the imidazole ring of AFB_1-N^7-Gua opens and results in the formation of a more stable product known as AFB_1-formamidopyrimidine (AFB_1-FAPY) which retains in the DNA for years with definite chromatographic characteristics (Carvajal et al. 2012). Under the low pH conditions, hydrolysis of the glycosidic bond results in the release of both AFB_1-FapyGua and AFB_1-N^7-Gua from the DNA (Coskun et al. 2018). The aflatoxin-N^7-Gua forms guanine (purine) to thymine (pyrimidine) transversion mutations in DNA which affects the p53 suppressor gene in the cell cycle. The p53 gene is important in preventing cell cycle progression when there are DNA mutations or signaling apoptosis. The transversion of base G to base T by AFB_1 occurs in the third position of codon 249, and similar mutations have been observed in hepatocellular carcinoma (HCC) in regions including East Asia and Africa having food contaminated with high levels of AFB_1 (Li et al. 1993; Mace et al. 1997). The serum AFB_1-albumin adduct is the marker of internal dose while the AFB_1-N^7- guanine is the biomarker of effect as the DNA adduct formation is the biochemical mechanism through which the AFB_1 expresses its carcinogenic potential.

Limited studies have investigated the presence of aflatoxin DNA adducts in humans. In a study from Mexico, the association between AFB_1-FAPY adduct and Human Papilloma Virus (HPV) types 18 and 16 quantified from DNA cervical scrapes from 40 females suffering from cervical cancer (CC) and 14 healthy females was determined. The mean levels of AFB_1-FAPY in the females with CC, HPV16, and HPV18 were 1025 pg/mg DNA, 1420 pg/mg DNA, and 630 pg/mg DNA, respectively, while it was only <2.6 pg/mg DNA in the healthy females ($p = 0.00006$). The findings revealed that in case of occurrence of AFB_1-FAPY adduct, the risk of CC increased sixfold with the odd ratio of 6.1 (95% CI: 1.4–25.4). In another study from Mexico, AFB_1-N^7 Gua was analyzed in the urine samples from 210 participants. The findings of the study revealed that concentration of AFB_1-N^7 Gua in the patients with viral cirrhosis (26%), alcoholic cirrhosis (10%), HBV (50%), HCV (16.6%), kidney failure (0.47%), chronic nonhepatic diseases (0%), and healthy patient (10%) was 0.4-323 ng/mg creatnine, 1-2 ng/mg creatnine, 1-43 ng/mg creatnine, 1-4 ng/mg creatnine, 0 ng/mg creatnine, 0 ng/mg creatnine, and 1 ng/mg creatnine with the occurrence frequency of 26%, 10%, 50%, 16.6%, 0.47%, 0%, and 10%, respectively (Alvarez-Banuelos et al. 2015). Egner et al. (2006) measured the level of AFB_1-N^7 Gua in the urine samples collected from 20 humans from the US region with acclaimed aflatoxin exposure. The AFB_1-N^7 Gua was found in around 80% of the studied samples with the average level of 0.28 pg/mg creatinine (2.9 pg/20 mL urine) and the range of $<$0.8-7.2 pg/20 mL. Mykkänen et al. (2005) studied 300 Chinese adults for their urinary concentration of AFQ_1, AFM_1, and AFB_1-N^7 Gua with the median concentration of 10.4 ng/ml, 0.04 ng/ml, and 0.38 ng/ml, respectively. On the other hand, AFB_1-N^7 Gua in all of the urine samples (total 113) of Brazilian adults was less than LOD (Jager et al. 2016).

Ochratoxin A

Ochratoxin A (OTA) is produced mainly by *Penicillium* and *Aspergillus* species (Varga et al. 2013). It has been observed to be present in cereals and cereal-based products, coffee, grapes, and nuts (Marin et al. 2013). The OTA is a hazardous fungal metabolite that exhibits nephrotoxic, carcinogenic, teratogenic, immunotoxic, and neurotoxic properties. This mycotoxin has also been categorized as a group 2B possible human carcinogen by IARC (Vidal et al. 2018b). Among various species, humans exhibit highest levels of OTA absorption ranging between 62 and 100% (Versantvoort et al. 2005).

Various putative biomarkers have been assessed in search of a suitable biomarker for OTA over a number of years leading to an improved assessment of risk linked through intake of this toxic compound via oral route or inhalation route of these natural toxins (Table 3). The occurrence of a chemically related DNA adduct is a better indicator of exposure and hence can be utilized as a biomarker (Pfohl-Leszkowicz and Manderville 2007). Contradictory reports have been published from different corners of the world about the occurrence of OTA-related DNA adducts. A few of the published studies are in favor of the presence of OTA-DNA adducts (Castegnaro et al. 2006; Mantle et al. 2010) and utilize their presence as indication of the OTA effect in BEN and related UTT patients (Pfohl-Leszkowicz and Manderville 2007), while some studies have proposed the contradictory results (Mally et al. 2004; Gross-Steinmeyer et al. 2002) and are in favor of oxidative stress instead of its relevance to a genotoxic mode of action for OTA.

In a study conducted in Egypt, fifty breastfeeding mothers and their exclusively breastfed infants were examined for the presence of OTA in serum (both of mother and infants) and mother milk samples. Among the analyzed samples, 36% samples were recorded positive for OTA. It was found that that the presence of OTA was linked with high levels of microalbuminuria and β2 microglobulin in urine samples. The higher levels of β2 microglobulin and microalbuminuria indicate the increased chances of kidney diseases in the people consuming OTA-contaminated diet (Hassan et al. 2006). Bernhoft et al. (2018) reported the impact of OTA-contaminated feed on the different clinical and biochemical parameters of *Salmo salar* (fish). Nonsignificant impacts of OTA were recorded for liver weight, but an increase in the levels of total protein, cholesterol, AST, alkaline phosphatase, and albumin was recorded after 3 weeks of OTA intake (0.2-2.4 mg/kg). In another study, conducted by Abdel-Wahhab et al. (2017), the rats ($n = 10$) were fed with OTA-contaminated diet. The results of the study indicated that the intake of OTA resulted in significant decrease in the levels of serum albumin and total proteins, significant increase in the levels of serum uric acid, urea, and creatinine, and decreased gene expression (GPx and SOD) and DNA fragmentation. In a study conducted by Zhu et al. (2016), three different groups of rat (each of 6 animals) including control, low dose (1 mg/kg), and high dose (4 mg/kg) were used to evaluate the impact of OTA on the health of animals. OTA intake led to reduced body weight, serum TP, AST, BUN and ALP levels, also increasing the mRNA expression of a few genes in kidneys. A strong link between OTA intake and kidney damage was established in this study.

Table 3 Ochratoxin (OTA) exposure, effected biological parameters, and health implications

Location	Type of sample / species	OTA exposure level	Number of samples	Effected biological parameters	Health implications	Reference
Egypt	Serum and milk of mothers and infants	Mother serum, mother milk, and infant serum, 4.28 ± 3.97, 1.89 ± 0.98, and 1.26 ± 1, respectively	50 (serum, mothers) 50 (serum, infants) 50 (milk)	Increased levels of β2 microglobulin and microalbuminuria	Increased risk of kidney diseases	Hassan et al. (2006)
Norway	Salmo salar (fish)	0.2–2.4 mg/kg	180	Increased levels of total protein, cholesterol, AST, alkaline phosphatase, and albumin	No clear health implications were recorded	Bernhoft et al. (2018)
Egypt	Rat	3 mg/kg diet	120	Decrease in the levels of serum albumin and total proteins, significant increase in the levels of serum uric acid, urea, and creatinine	Kidney damage and nephrotoxicity	Abdel-Wahhab et al. (2017)
China	Rat	Low dose (1 mg/kg), high dose (4 mg/kg)	18	Increased levels of ALP, AST, BUN, TP, and mRNA expression of some genes increased in kidney but decreased in liver	Kidney damage and oxidative damage in kidney and liver	Zhu et al. (2016)
China	Human kidney tubular epithelial cells (HK-2)	2, 4, and 8 μM	Not given	Increased levels of reactive oxygen species and malondialdehyde. Decreased activity of glutathione and superoxide dismutase	Apoptosis in HK-2 cells	Song et al. (2021)

Song et al. (2021) evaluated the impact of OTA introduction on human kidney tubular epithelial cells (HK-2). The findings indicated that the increased levels of OTA lead to apoptosis of HK-2, decreased levels of glutathione and superoxide dismutase activities, and increased levels of malondialdehyde and reactive oxygen species. In short, the exposure of OTA in humans or animals may lead to severe damage of kidney and nephrotoxicity, and the possible biomarkers of effect may be increased levels of β2 microglobulin and microalbuminuria, increased levels of reactive oxygen species, total protein, cholesterol, AST, alkaline phosphatase, and decreased activity levels of glutathione and superoxide dismutase.

Deoxynivalenol

Deoxynivalenol (DON) is a type B trichothecene mycotoxin produced by fungus Fusarium when they contaminate wheat, barley, or corn. DON is among one of the most frequently occurring fungal contaminants of food and feed globally. Owing to its emetic effects, it is also known as "vomitoxin" and is associated with human gastroenteritis. Studies of animal models have shown that acute poisoning of DON causes emesis while chronic exposure results in growth retardation and immunotoxicity. The chronic exposure to DON may also impair reproduction and development due to maternal toxicity. The effects on physiology include alteration of neuroendocrine signaling, altered gut integrity, induction of proinflammatory gene, and disruption of growth hormone axis. Additionally, DON is responsible for ribotoxic stress at cellular level consequently affecting the synthesis of macromolecules, cell signaling, differentiation, proliferation, and death. The DON-induced toxicity varies based on the specie as well as the exposure to other mycotoxins. Studies carried out on human intestinal mucosal cell lines indicated that DON-induced toxicity is done by altering the intestinal transporter's activity, thus causing the malabsorption of nutrients, and the major pathway of injury happens by the suppression of protein synthesis and reduction in the molecular constituents of intracellular junctions (claudina-4) (for details, please review Silva et al. 2021).

Glucuronidation, oxidation, sulfation, and sulfonation are the main biotransformation pathways followed by DON. Ingested DON is usually excreted in the urine in the form of free DON and DON-glucuronide (DON-GlcA), including DON-15-GlcA and DON-3-GlcA. The free-DON and DON-glucuronide, in urine, collectively amounting to total urinary DON (T-DON) were suggested as exposure biomarkers for DON after experiments on rat models (Meky et al. 2003). DON **3**-glucuronide (GclA), DON-15-GlcA, and DON-7-GlcA are the main metabolites of DON in humans. The biomarkers of DON appear to be closely associated to age, gender, region, and even dietary habits. Besides free-DON and DON-3-GlcA, DON-3-sulfate was recognized as potential novel biomarker of DON in humans (Warth et al. 2016). DON-sulfate has been found to increase multiplication of cancerous cell lines, including human colon epithelial cells (HCEC-1CT), human HT-29 colon carcinoma cells, and T24 bladder cancer cells (Warth et al. 2016). Based on the various toxicological effects of DON, other potential biomarkers of effect might include proinflammatory cytokines,

suppressors of cytokine signaling (SOCS), and IGF1. However, the limitations of proinflammatory cytokines include their brief expression and nonspecificity as they can be produced by other mediators as well. Additionally, liver SOCS3 mRNA or protein may also prove to be reliable indicators of DON effect as these can be well detected after the onset of cytokine decline (Pestka 2010).

Fumonisins

Fumonisins are fungal secondary metabolites produced by *F. verticillioides*, *F. proliferatum*, and *F. nygama*. This category of mycotoxins has been observed mainly in maize (Marin et al. 2013). Twelve forms of FB are described among which fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), and fumonisin B_3 (FB_3) are the most important. The FB_1, however, is the most critical one owing to its high toxicity and has been classified in group 2B of potential human carcinogens by IARC (Vidal et al. 2018b). Due to this reason, JECFA has recommended the reduction in FB exposure, especially in regions where maize is consumed as major dietary staple food (JECFA 2017). The FB_1 comprises of approximately 70% of total naturally occurring FBs in food and feed samples contaminated with fungus (Kamle et al. 2019). The animals exposed to FB_1 may exhibit both acute as well as chronic symptoms. The FB_1 is nongenotoxic; however, it is an initiator of cancer.

The major organs affected by FB_1 toxicity include liver, kidney, and intestine (Voss et al. 2007; Bouhet and Oswald 2007). It was revealed that equine leucoencephalomalacia occurring usually in horses is caused by consumption of FB_1-contaminated maize (Haliburton and Buck 1986). It has also been reported that consumption of maize infected by *F. verticillioides* is responsible for porcine pulmonary edema (PPE) (Marasas 2001). Additionally, the ingestion of fumonisin-contaminated food by pregnant women also results in neural tube defects in the developing fetus (Missmer et al. 2006; Marasas et al. 2004) and causes embryotoxicity as well (Sadler et al. 2002). The dose above threshold levels may also result in fetal death. Although the FB_1 exposure is associated with esophageal and liver cancer, these associations have not been validated and are only based on observational studies (Alizadeh et al. 2012; Sun et al. 2011). Although no direct evidence of fumonisin toxicity are reported, its prolonged exposure may lead to cancer and birth defects in humans (Liverpool-Tasie et al. 2019).

The mechanism of toxicity exhibited by FB_1 is attributed to its structural similarity to sphingoid bases (Wang et al. 1991). The FB_1 displays structural similarities to sphingoid bases consequently acting as a competitive inhibitor of ceramide synthase (Riley et al. 2012; Marasas et al. 2004). It is noteworthy that de novo synthesis pathway of sphingolipids is highly dependent on the action of ceramide synthase. Therefore, FB contamination is a matter of concern due to its interference with sphingolipid metabolism ultimately leading to severe health concerns. The process starts with the condensation of serine and palmitoyl-CoA catalyzed by serine palmitoyl transferase forming 3- ketodihydrosphingosine which is further reduced to sphinganine. Subsequently, it is N-acylated by ceramide synthase to generate

dihydroceramide [dhc] that is converted to ceramide by the action of dhc-desaturase. However, in the case of fumonisin exposure, FB_1 restricts the process of N-acylation by inhibiting ceramide synthase (Voss et al. 2006; Zitomer et al. 2009). This inhibition consequently results in accumulation of intracellular sphinganine which can lead to oxidatively generated DNA damage, sphingomyelin depletion, and altered function of sphingolipids as second messengers. Also, the increase in the rate of increase of sphinganine metabolites in comparison to the cell's ability to transform them to their sphinganine base-1-phosphate may disrupt the intracellular levels of sphingolipids metabolites that induce or prevent apoptosis thus leading to increased toxicity (Merrill et al. 2001; Wangia et al. 2019).

The free sphingoid bases (sphinganine [Sa] and sphingosine [So]) and the Sa/So ratio in urine and blood have been validated as reliable biomarkers of effect for fumonisin in both humans and animals (Baldwin et al. 2011; Wangia et al. 2019), as presented in Table 4. The Sa/So ratio is dependent upon dose and the specie of the animal. Additionally, the elevation in free sphinganine and the Sa/So ratio are specific to ceramide synthase inhibition (Grenier et al. 2015). A rapid increase in free sphinganine, which is a substrate for de novo biosynthesis of dihydroceramide, occurs when the enzyme ceramide synthase is inhibited as a result of FB ingestion. Furthermore, sphingosine is formed when the sphinganine in dihydroceramide is desaturated, and thus free sphingosine is produced only as a result of the much slower conversion of ceramide and more complex sphingolipids. Due to this reason, the free Sa/So ratio increases when ceramide synthase is inhibited in animal studies.

In a cross-sectional study conducted in two high-risk populations of China, 83.1% of the studied participants were found to have urinary-free FB_1 with the median level of 3.91 ng/mg creatinine (Huaian subjects) and 0.31 ng/mg creatinine (Fusui subjects). However, the median serum levels of Sa/So ratio 0.41 and 0.78 in Huaian and Fusui subjects, respectively, were not correlated with dietary FB_1 exposure (Xu et al. 2010). In another study from Kenya, a total of 284 children were analyzed for the urinary concentration of Sa, So, and Sa:So ratio. Around 98.94% and 95.07% samples of urine were found to contain So and Sa, respectively, with the creatinine adjusted average value of 1.23 nM for Sa, 4.99 nM for So, and 0.296 for Sa/So. These studies revealed that although Sa, So, and Sa/So are suggested biomarkers of effect for FB_1, some studies have proposed contrastingly, proposing that these parameters are not sensitive enough to be used as biomarkers for FB (Van Der Westhuizen et al. 2013). However, it was further showed that increase in free Sa and the Sa/So ratio can only be detected in urine and blood when the fumonisin intake is very high (Xue et al. 2015).

Zearalenone

Zearalenone (ZEN) is a mycotoxin that is produced by several species of *Fusarium* fungi mainly by *Fusarium graminearum*. Concentration of ZEN is usually low in the on-field grains (before harvesting), and the higher levels of ZEN are usually reported in the stored cereal grains with moisture level higher than 30 – 40%. ZEN mainly

Table 4 Fumonisin (FB) exposure, effected biological parameters, and health implications

Location	Type of sample / species	FB exposure level	Number of samples	Effected biological parameters	Health implications	Reference
Egypt	Serum samples of children and adults	Adult with hemodialysis therapy (RDT), children with RDT, adult with glomerulonephritis (GN), and children with GN was 1.34, 0.93, 1.56, and 0.30 ng/ul, respectively	40 individuals (20 children and 20 adults) with renal disease and 10 healthy as control	30% patients with end-stage renal disease and 30% patients with different glomerulonephritis, 22.5% having delayed visual evoked potential, were positive with FB_1	Hyperparathyroidism and other neurological defects	Saadi et al. (2002)
Brazil	Wistar rats	Low dose (1 mg/kg), high dose (4 mg/kg)	18	Decreased metabolic activity of myenteric neurons, reduced neuronal density of metabolically active neurons, and the cell body area nitrergic neurons	Reduced respiratory metabolism of mitochondria in brain and liver	Sousa et al. (2020)
Germany	Pigs	80.9 mg FB_1 + 33.2 mg FB_2 + 6.1 mg FB_3/kg feed	31	Increased sphinganine/ sphigosine ratio in the blood	Disruption of sphingolipid metabolism	Schertz et al. (2018)
West Africa	Urine, buccal cells, and serum samples of adults	FB_1 + FB_2: 0.324 mg/kg of maize paste	284	Increased sphinganine/ sphigosine ratio in the blood	Disruption of sphingolipid metabolism	Nikiema et al. (2008)

effects the reproductive system especially of females. The International Agency for Research on cancer has classified ZEN as group 3 carcinogen (IARC 1993). The metabolism of ZEN mainly occurs in liver by the action of cytochrome P450 enzymes; however, they may also be metabolized to some extent in the intestine. ZEN is reported to metabolize into α-ZEL and β-ZEL after the degradation of keto group on carbon number 8, although both of the earlier mentioned metabolites may be produced directly by Fusarium species but in very low concentrations. In mammals, ZEN is reported to metabolize via two major pathways: The first is hydroxylated breakdown of ZEN into α-ZEL and β-ZEL, and this reaction is catalyzed by 3α- and 3β-hydroxy-steroid-dehydrogenases; the second is ZEN conjugation with glucuronic acid, and this reaction is catalyzed by uridine diphosphate glucuronyl transferases. Significant differences in ZEN metabolism are reported in different species of animals (Gupta et al. 2018; Zhang et al. 2018).

ZEN is reported to have strong estrogenic activity. ZEN concentration of around 1 ppm is enough to cause hyper estrogenic condition, and further higher doses may lead to miscarriages, complications in conception, enlargement of vulva, and hormonal disturbances. The xenoestrogen activity of ZEN is mainly due to its structural resemblance with estradiol, i.e., a hormone produced by ovaries (Rogowska et al. 2019). ZEN may adversely affect the uterus by changing the morphological structure of uterus tissues or by decreasing the secretion of luteinizing hormone and progesterone. A number of studies have confirmed that ZEN negatively affects the health of domestic animals and some laboratory animals. Increased doses of ZEN in animal feed may lead to abnormal changes in serum levels of estradiol and progesterone, reduction in the weight of pituitary glands and thyroid glands, and decreased level of fertility with less chances of teratogenic toxicity (Zinedine et al. 2007; Zhang et al. 2018). Zwierzchowski et al. (2005) reported abnormal changes in the growth and maturation of ovarian sacs/follicles in ZEN feed Gilts (200 μg/kg b.w.). ZEN and its metabolites are also reported to have immune toxic impacts and can alter the viability of immune cells; their proliferation can disturb cell cycle and may also disturb immune cell functions such as active molecule generation potential and inflammatory responses. Exposure to ZEN may also lead to cell death (necrosis), apoptosis, and malfunctioning of cells (Bulgaru et al. 2021).

Concluding Remarks

The presence of mycotoxins in food is a primary global concern and an inevitable problem to food safety and security. Because of the high health risks associated with dietary mycotoxins, regulatory agencies around the world have adopted regulations on susceptible foods, mainly for AFs, ZEN, OTA, FBs, and DON. Traditional exposure assessments of humans to mycotoxins have been conducted using the occurrence data of these contaminants in foods and their consumption patterns. However, the use of biomarkers detected in biological fluids has provided a more reliable and real estimation of individual exposure to mycotoxins, especially AFs and DON. Further studies are needed to validate suitable biomarkers that provide a

clear association with the adverse health effects caused by other regulated mycotoxins, such as OTA, ZEN, and FBs.

Applications to Prognosis, Other Diseases, or Conditions

Mycotoxin biomarkers have been proven to be useful to determine the actual exposure to mycotoxins, mainly through the consumption of contaminated foods. The toxicokinetic data of mycotoxins in humans and animals indicate suitable biomarkers that provided significant associations between dietary exposure to mycotoxins and human disease, especially regarding the AFs and liver cancer. The risk of esophageal squamous cell carcinoma also correlated with the occurrence data of AFB_1 and FB_1 biomarkers found in body fluids in China (Xue et al. 2019). The free-DON and DON-glucuronides found in urine account for the total urinary DON, which has been recommended as suitable exposure biomarkers to quantitatively assess the dietary exposure to DON. Thus, human biomonitoring through validated biomarkers has potential applications in regional and national risk assessments of mycotoxins, as well as in reevaluations of the effectiveness of tolerance limits adopted for these contaminants in foods.

Mini-Dictionary of Terms

- Biotransformation: The chemical transformation of an organic compound within the organism, usually carried out by enzymes in the liver, which may originate different substances derived from the parent compound.
- Exposure: The amount of a chemical substance that has been ingested through food consumption, usually expressed as the daily intake of the substance.
- Mycotoxin: A toxic secondary metabolite produced by certain species of molds during growth on foodstuffs.
- Regulated mycotoxins: A group of major mycotoxins in terms of toxicity and worldwide occurrence, for which maximum permitted levels have been established in several countries.
- Toxicokinetic: The description of the rate at which a chemical substance enters the body, and the subsequent events that take place, thereafter, including metabolization, distribution, accumulation, or excretion.

Key Facts of Mycotoxin Biomarkers

- Biomarkers can accurately evaluate the exposure to chemical compounds.
- Urinary and serum biomarkers indicate the level of exposure to certain mycotoxins, such as AFs and DON.
- Urinary AF-N^7-guanine is a predictive biomarker of hepatocellular carcinoma induced by AFs.

- AFM_1 in urine and serum AFB_1-lysine are biomarkers of short-term and of long-term exposures to AFs, respectively.
- Suitable biomarkers for mycotoxins OTA, ZEN, and FBs still require validation.

Summary Points

- Dietary exposure to mycotoxins is a primary global concern in terms of food safety and security.
- Human exposure assessments to mycotoxins are essential to protect consumers and evaluate the effectiveness of regulations for these food contaminants.
- Urinary and serum biomarkers are effective for quantifying the human exposure to certain mycotoxins at the individual level.
- Validated biomarkers for AFs are AFM_1, AF-N^7-guanine, and AFB_1-lysine, while free-DON and DON-glucoronides (DON-**3**-GclA, DON-15-GlcA, and DON-7-GlcA) are suitable biomarkers of DON exposure.
- Further studies are needed to validate suitable biomarkers for OTA, ZEN, and FBs.

Cross-References

► DNA Adducts as Biomarkers in Toxicology
► LC-MS-Based Metabolomics in the Identification of Biomarkers Pertaining to Drug Toxicity: A New Narrative

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14 Metabolomics Profiling of Di-(2-propylheptyl) Phthalate (DPHP) Biotransformation Products as Exposure Markers: Analytical Strategy and Application

Shih-Wen Li, Chih-Wei Chang, Yuan-Chih Chen, Jing-Fang Hsu, and Pao-Chi Liao

Contents

Abstract

Phthalates are widely used in the manufacture of plastics and personal care products, and humans are easily exposed to phthalates through the usage of these products. Phthalates and di-(2-propylheptyl) phthalate (DPHP), the alternative plasticizer to replace traditional phthalates, are toxicants and their half-life of the original chemical forms in humans is less than 24 h. Therefore, the biological monitoring of phthalates and DPHP are commonly performed by measuring the

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corresponding metabolites instead of the original chemical form. This chapter presents the strategy of combining high-resolution mass spectrometry-based metabolomics method and dose-response study in laboratory animals to discover DPHP biotransformation products as exposure markers. This strategy included screening biomarkers, verification of dose-response relationships in laboratory animals, and application in human subjects. First, the multivariate data analysis method (orthogonal partial least squares-discriminant analysis OPLS-DA and mass defect filter, MDF) and isotope tracing method (signal mining algorithm with isotope tracing, SMAIT) were used to screen and find meaningful signals in mass spectrometry (MS) dataset generated from urine samples collected from DPHP-administered rats or in vitro-incubation sample of DPHP. Next, the meaningful MS signals were verified as exposure marker candidates by assessing dose-response relationships in an animal feeding study. Finally, the exposure marker candidates are applied in human subjects and identified the chemical structures. A biotransformation product of DPHP, mono-(2-propyl-7-dihydroxy-heptyl) phthalate, was suggested as a DPHP exposure marker for general human exposure assessments.

Keywords

Phthalate · DPHP · Metabolite · Biotransformation · Exposure marker · Biomarker · OPLS-DA · SMAIT · MDF

Abbreviations

BBP	Butyl-benzyl phthalate
cx-MEPP	Mono-(2-ethyl-5-carboxypentyl) phthalate
cx-MPBP	Mono-(2-propyl-4-carboxybutyl) phthalate
cx-MPHxP	Mono-(2-propyl-6-carboxyhexyl) phthalate
DBP	Di-n-butyl phthalate
DEHP	Di-(2-ethylhexyl) phthalate
DIA	Data-independent acquisition
DINP	Di-iso-nonyl phthalate
Di-OH-MPHP	Mono-(2-propyl-7-dihydroxyheptyl)-phthalate
DPHP	Di-(2-propylheptyl) phthalate
FT-ICR	Fourier transform ion cyclotron resonance
GC	Gas chromatography
IP	Isotopic pair
IPRR	IP response ratio
LC-HRMS	Liquid chromatography-high resolution mass spectrometry
MDF	Mass defect filter
MPHP	Mono-(2-propylheptyl)-phthalate
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MW	Molecular weight

OH-MPHP	Mono-(2-propyl-6-hydroxyheptyl) phthalate
OPLS-DA	Orthogonal partial least squares-discriminant analysis
oxo-MPHP	Mono-(2- propyl-6-oxoheptyl) phthalate
PVC	Polyvinyl chloride
Q-TOF	Quadrupole-TOF
RTW	Retention time window
S/N ratio	Signal-to-noise ratios
SDS	Sodium dodecyl sulfate
SMAIT	Signal mining algorithm with isotope tracing
TOF	Time of flight
ΔMZ	Mass shift
ΔRT	Retention time difference

Introduction

Phthalates are widely used in industry, plastics, construction materials, and personal care products, so humans are frequently exposed to phthalate compounds. More than 18 billion pounds of phthalates are used every year. Phthalates are used to be plasticizers in the manufacture of polyvinyl chloride (PVC) products and solvents or fixatives in fragrances (Bagchi et al. 2018; Weinstein et al. 2017). Phthalates are lipophilic and can be absorbed via inhalation, ingestion, and direct skin contact. Phthalates can rapidly be metabolized into their respective monoesters body to increase water solubility through phase I biotransformation in the human (Latini 2005; Frederiksen et al. 2007; Marklund et al. 2010; Fierens et al. 2012). In addition, the monoesters could conjugate the chemical to a polar glucuronide, result in increasing the aqueous solubility and promoting excretion (Zhang et al. 2009; Marklund et al. 2010). In recent years, exposure to phthalates has attracted a large number of concerns because of their adverse effects on human health, especially on the reproductive system (Lin et al. 2010). For example, phthalates are detected in human breast milk and can cause incomplete virilization in newborn boys (Main et al. 2006). The free testosterone of serum is decreased in the workers that were exposed to high doses of di-n-butyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP) in China (Pan et al. 2006). In addition, DEHP, DBP, di-iso-nonyl phthalate (DINP), and butyl-benzyl phthalate (BBP) are scrutinized due to the endocrine-disrupting effects (Hsu et al. 2019). Therefore, the use of traditional phthalates is prohibited.

Di-(2-propylheptyl) phthalate (DPHP) is used as an alternative plasticizer to manufacture PVC. DPHP could be easily released from plastic products, because it is not chemically bound in the polymer (EC JRC 2003; Johnson et al. 2010). Thus, the risk of human exposure to DPHP may be high. It was reported that the detection rate of DPHP in Germany has risen from 3.3% in 2009 to 21.7% in 2012, and the global consumption of DPHP is continuously increasing over time (Schütze et al. 2015). Previous studies reported that DPHP does not affect fetal testicular testosterone production (Furr et al. 2014), but experimental evidence demonstrates that

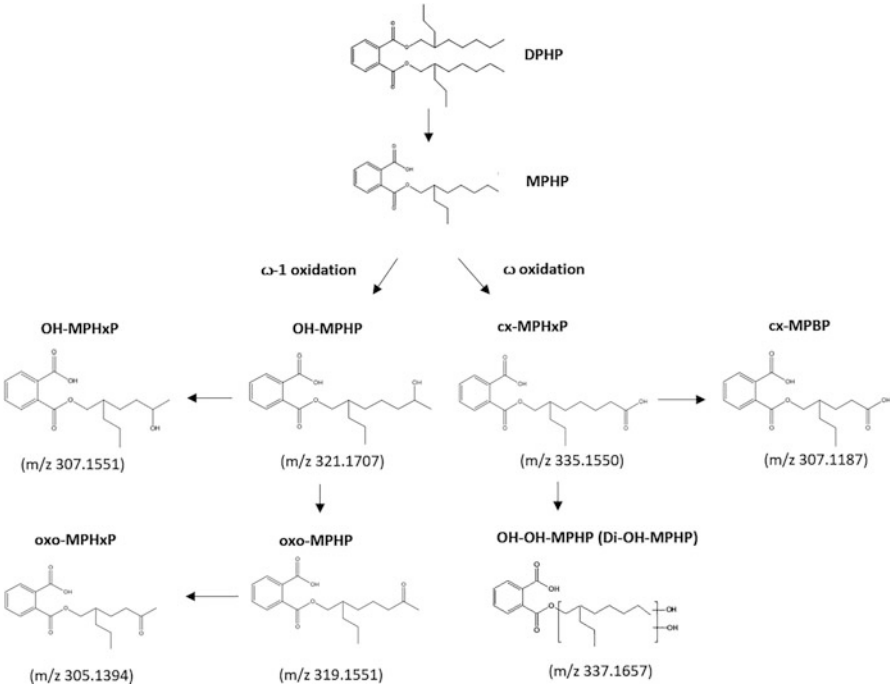


Fig. 1 Proposed biotransformation products of DPHP. (The data comes from the literature reported by Hsu et al. (2019) and Shih et al. (2019b))

DPHP is a subchronic toxicant (CPSC 2011). Some adverse effects were detected in rats exposed to DPHP, including decreasing body weight, increasing liver weight, altered thyroid and pituitary functions, and histopathology changes in adrenal, liver, and soft tissue histopathology (BASF 2009; CPSC 2010). Four biotransformation products have been postulated as DPHP biotransformation products in humans, including mono-(2-propylheptyl)-phthalate (MPHP), mono-(2-propyl-6-oxoheptyl) phthalate (oxo-MPHP), mono-(2-propyl-6-hydroxyheptyl) phthalate (OH-MPHP), and mono-(2-propyl-6-carboxyhexyl) phthalate (cx-MPHxP) (Fig. 1) (Wittassek and Angerer 2008; Gries et al. 2012). A human volunteer study showed that urinary elimination half-lives of oxo-MPHP, OH-MPHP, and cx-MPHP are less than 8 h, and within 48 h after oral application, only 24% of the applied doses were eliminated in the urine as the three DPHP biotransformation products (Leng et al. 2014; Klein et al. 2018). Hence, an effective method for discovering exposure markers (original forms or biotransformation products) with a longer half-life and a higher amount in humans is essential.

Mass spectrometry (MS) plays a critical role in metabolomics research, especially in endogenous metabolites and the biotransformation products of xenobiotics detection and identification in complex matrices (Cooks et al. 2015; Hsu et al. 2017). Endogenous metabolites in biological samples are often present at low levels and

have an excessive matrix background (Hsu et al. 2017). Despite the complexity of endogenous metabolites/biotransformation products and the matrix environment, the availability of high-resolution mass spectrometry (HRMS) with improved mass accuracy has dramatically improved the applicability and productivity of metabolite identification processes. However, handling complex MS datasets to provide positive metabolite identification is still a challenge. Therefore, it is necessary to develop an efficient strategy for metabolite signal filtering (Forsberg et al. 2018; Lu et al. 2017). Multivariate analysis methods are one of the approaches to finding meaning peaks in metabolomics datasets in which the peaks contributing the most to variations or separation are identified for further targeted analysis, such as principal component analysis (PCA), partial least squares projection to latent structures (PLS), and orthogonal partial least squares discriminant analysis (OPLS-DA). This chapter presents the strategy of combining high-resolution mass spectrometry-based metabolomics method and dose-response study in laboratory animals to discover DPHP biotransformation products as exposure markers.

DPHP Exposure Marker Discovery

Workflow of DPHP Exposure Marker Discovery

Figure 2 outlines the general workflow for MS-base exposure marker discovery, includes sample preparation and MS data acquisition, MS data processing, and chemical structure identification. In addition, the biospecimen collected from the DPHP-administered laboratory animal model and in vitro-enzyme incubation sample of DPHP can be used as the target samples. Here report the strategy of combining high-resolution mass spectrometry-based metabolomics method and dose-response study in laboratory animals to discover DPHP biotransformation products as exposure markers. This strategy included screening biomarker candidates from MS data, exposure marker candidates verification of dose-response relationships in laboratory animals, and application in human subjects. After the peak table generated from the biological samples by HRMS, the multivariate data analysis method (OPLS-DA, MDF, etc.) and isotope tracing method (signal mining algorithm with isotope tracing, SMAIT) were used to screen and find meaningful peaks in the MS dataset. Next, the meaningful MS peaks were verified as exposure marker candidates by assessing dose-response relationships in an animal feeding study. Finally, the exposure marker candidates are applied in human subjects and identified the chemical structures.

Sample Preparation and LC-MS Analysis

Various specimens, such as urine, blood, hair, etc., have been exploited for exploring biological exposure. Blood samples usually have to deproteinize by precipitating with methanol, acetone, acetonitrile, or hydrochloric acid. Urine samples, which

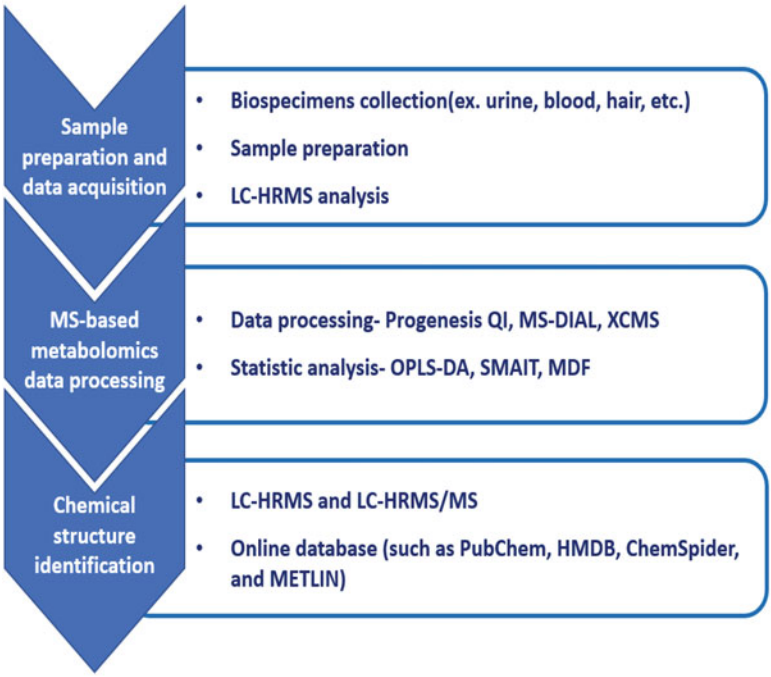


Fig. 2 Workflow for the discovery of exposure markers. Overall, three steps for exposure marker discovery, including sample preparation and data acquisition, metabolomics data processing, and chemical structure identification

commonly add an antibacterial agent like sodium azide to prevent microbial contamination after collection, are hydrolyzed with β-glucuronidase and sulfatase to remove the conjugates of phase II metabolites (Marklund et al. 2010). To reduce interference and preconcentration of the analytes, biospecimens are prepared by common procedures, such as solid-phase extraction, liquid-liquid extraction, and QuEChERS. Hair samples are washed with sodium dodecyl sulfate (SDS), followed by deionized water three times in the ultrasonic bath, and then air-dried (Shih et al. 2019a). The hair samples are cut into 1–2 mm snippets and homogenized. Then, a hair sample (about 50–100 mg) is used to extract the analytes by a methanol and trifluoroacetic acid mixture with sonicated. The extracted sample is concentrated to dryness by nitrogen stream evaporation or speed vac and then dissolved in formic acid (Shih et al. 2019a, b).

LC and gas chromatography (GC), both highly efficient for separation and often hyphenated with HRMS, identify and quantify components of a highly complicated mixture, such as endogenous metabolite detection and identification. HRMS, including the time of flight (TOF), Orbitrap, magnetic sector, and Fourier transform ion cyclotron resonance (FT-ICR), have highly increased the accuracy and selectivity of mass spectrometry, whereas LC is generally coupled with quadrupole-TOF (Q-TOF) and Orbitrap to be used in liquid form samples. The advantages of HRMS are fast

scan rate, high mass accuracy, excellent resolution, superior sensitivity, and multiple-stage mass spectrometry scanning (MS/MS). In addition, HRMS can provide high resolution (>10,000 at full-width at half-maximum) of ion measurements and accurate mass (<5 ppm deviation) capabilities that can determine the elemental composition of metabolite ions and their fragments.

MS Data Processing Approaches

The MS data processing can include generating a peak table, statistical analysis, and structure identification. For peak table generation, a great majority of the software can be used, such as XCMS (Smith et al. 2006; Tautenhahn et al. 2012), MS-DIAL (Tsugawa et al. 2015), Progenesis QI, etc. In addition, several strategies or statistical analysis methods have been used to screen and filter the meaning peaks from LC-HRMS data. In this chapter, three approaches, orthogonal partial least squares-discriminant analysis (OPLS-DA), the signal mining algorithm with isotope tracing (SMAIT), and mass defect filter (MDF), are used to identify the candidates of DPHP biotransformation products.

Peak table generation. Because of accurate mass measurement and comprehensive signal recording, HRMS is widely used in many laboratories, untargeted analysis becomes practical. The peak table generation is the first step in the typical untargeted analysis process. MS data processing requires processing more than 10,000 mass spectral signals in a complex HRMS dataset for untargeted analysis, so data processing automation is essential. The chromatographic peaks in the raw data are automatically integrated and aligned between samples. A great majority of the software, such as XCMS (Smith et al. 2006; Tautenhahn et al. 2012), MS-DIAL (Tsugawa et al. 2015), Progenesis QI, etc., can further convolute to group peaks with different charge states or ion adduct types generated by the same compound into a "feature." Among them, MS-DIAL and XCMS are open sources, as well as Progenesis QI is commercial software. In addition, MS-DIAL implements a new deconvolution algorithm for data-independent acquisition (DIA) datasets in LC-MS/MS, whereas XCMS Online can process data format from various instrument vendors.

Feature filter. Various methods have been developed to objectively search/filter LC-HRMS data to facilitate target peak detection. This chapter introduces the three methods of OPLS-DA, SMAIT, and MDF. OPLS-DA is one of the multivariate analysis methods. It utilizes multivariate data to discriminate between two groups and is based on the development of the PLS method (Worley and Powers 2013). It is a distinguished sample classification to help identify possible markers. The easy interpretability of OPLS-DA modeling with a dimensionality reduction and data fusion step are the advantages of OPLS-DA (Boccard and Rutledge 2013). Figure 3 shown an example of result figures of OPLS-DA. The OPLS-DA score plot shows the variation between the peak profiles of the two different groups with different treatments. Each dot indicates a score value for a result from a sample. The S-plot shows the direction of the hyperplane relative to the original X variable, which can

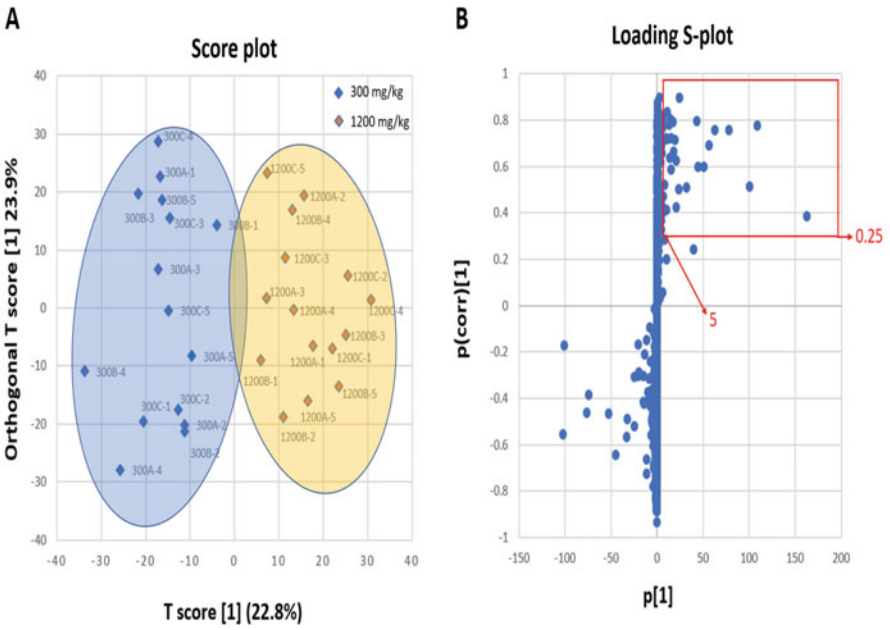


Fig. 3 Score plot and S-plot of OPLS-DA multivariate data analysis. (**a**) The OPLS-DA score plot shows the variation between the metabolite signal profiles of the two groups of DPHP exposure doses. Each point indicates a score value for a result from an animal. (**b**) The OPLS-DA loading S-plot shows the relative contributions of the signal variables to the clustering of rats dosed with 300 and 1200 mg/kg DPHP. Each circle indicates the loading value of a signal. (From the literature of Hsu et al. (2019))

well summarize the influence of the variable on the pattern (Hsu et al. 2019). S-plot shows that the covariance (P[1]) and correlation (P(corr)[1]) are the covariance and correlation of T score [1] and signal abundance, respectively. Thus, S-plot can filter the interesting signals to identify the candidates for DPHP exposure markers. The OPLS-DA analysis is free for use on MetaboAnalyst 5.0 website.

SMAIT is an algorithm based on the isotope tracing concept and used to filter target biotransformation products of xenobiotics (Lin et al. 2010). It contains three steps, including isotopic pair (IP) finding, IP response ratio (IPRR), and IPRR correlation analysis (Fig. 4) (Hsu et al. 2017). IP finding is used to find the signals with mass shifts in samples with varying isotope-labeled concentration ratios by performing the signal processing on LC-MS signal peaks extracted by peak extraction computational tools. Sample mixtures with varying isotope-labeled concentration ratios (naïve: isotope-labeled = 3:7, 4:6, 5:5, 6:4, and 7:3, respectively) are incubated with the liver enzyme to generate metabolites and then analyzed by LC-HRMS. The mass shift (ΔMZ) within the user-defined RT difference (ΔRT) is found between the native and isotope-labeled peak doublets in the hierarchical peak list of the isotope pair. If the alternation of the RTs and mass between the two peaks is less/equal than a given ΔRT and a given ΔMZ, these peaks are judged to be IP. In the

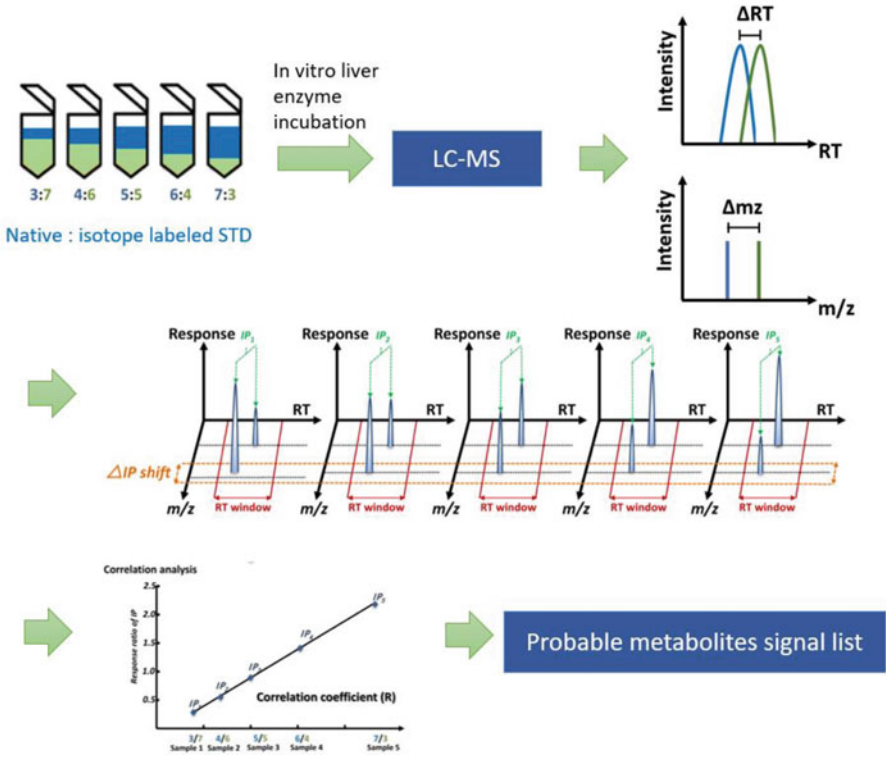


Fig. 4 The analytical approach of the SMAIT. There are three steps in the SMAIT approach, including isotopic pair (IP) finding, IP response ratio (IPRR), and IPRR correlation analysis. (From the literature of Hsu et al. (2017))

IPRR step, the IPs are located in five samples with different ratios (native: isotope-labeled) that give the response ratios correlated with the expected concentration ratios. Because the RT shifts may cause the loss of IP for a specific concentration ratio and raise the difficulty of excavating the metabolite signal using the SMAIT strategy, the user-defined sliding RT window (RTW) is similar to the RT tolerance, is used to replace the peak alignment process. The peak values of each sample in RTW are grouped to calculate Pearson's correlation coefficients. The RTW scan depends on the peaks in five UPLC-HRMS datasets with the different ratios and finds the IP combinations and their correlation coefficients. In the step of IPRR correlation analysis, this analysis locates the IP that gives a response ratio related to the expected concentration ratio. It is supposed that the high correlation coefficients of the IPs have a higher probability of containing probable metabolite signals.

MDF technique was first introduced and utilized for the identification peak of drug biotransformation products in 2003. It is developed to detect xenobiotics' endogenous metabolites and biotransformation products in complex matrixes using a high mass accuracy instrument, HRMS (Zhang et al. 2003; Zhu et al. 2006). The target compound

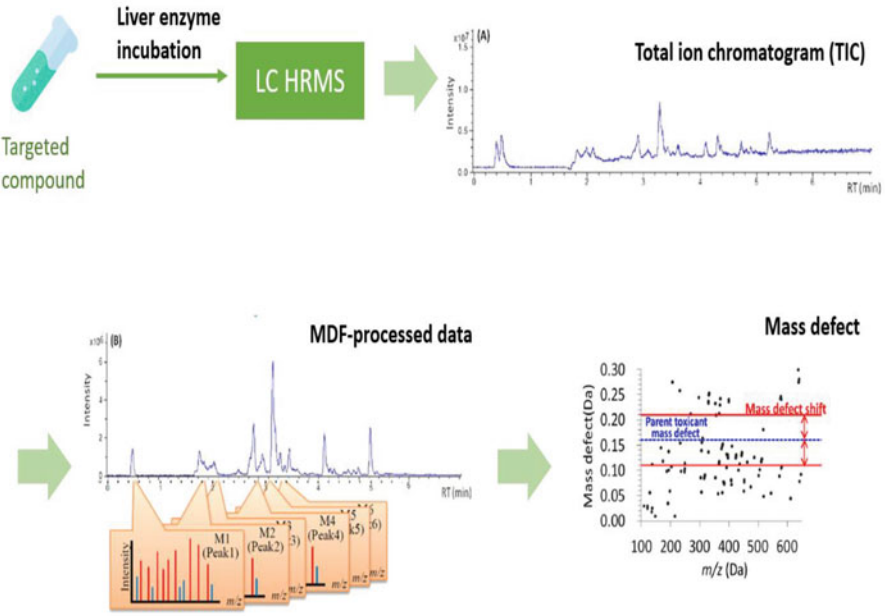


Fig. 5 The workflow of MDF. There are three steps to identify the biotransformation products, including in vitro incubation with liver enzyme, LC-HRMS analysis, and MDF-processed data. (From the literature of Hsu et al. (2017))

is incubated with the liver enzyme to obtain the biotransformation product mixture and then measurement by LC-HRMS, MS data processed by MDF software according to the defined mass defect change from the target compound's mass. Figure 5 is a schematic diagram for the MDF concept process. Each parent compound has a mass defect associated with the metabolites of phase I and phase II. Because many parent compounds structures of natural metabolites typically remain unchanged, the mass defect change of metabolites associated with their parent compounds situates within a relatively narrow range (usually within ± 0.050 Da). Depending on the parent compound's molecular weight (MW), the MW range of these metabolites can be estimated to filter off the ions that go beyond the expected MW range. The signals of mass defect change within the boundaries are retained by MDF, whereas the signals out of the mass defect shift are excluded. MDF-processed chromatogram automatically produces after processing, and the possible metabolites of target compounds with signal-to-noise ratios (S/N ratio) greater than 3 in the chromatogram processed can be obtained. This data processing approach of MDF permits users to concentrate on the analysis of potential chemical metabolite candidates.

Dose-Response Verification

A suitable exposure marker can increase the abundance correspondingly as the concentration or time of exposure increases. To verify the biotransformation

products candidates of DPHP identified by the workflow (Fig. 2), the different doses of DPHP are administered to experimental animals further to perform the dose-response validation of exposure marker candidates. The biotransformation products candidates of DPHP that can show a dose-response relationship under different DPHP exposure doses were validated as exposure marker candidates of DPHP.

Chemical Structure Identification of DPHP Exposure Markers

The chemical structure of DPHP exposure marker candidates is identified based on their accurate masses and product ion spectra in negative mode obtained by LC-HRMS and MS/MS. In addition, the chemical structures of the exposure marker candidate peaks were identified by online databases (such as PubChem, HMDB, ChemSpider, and METLIN) (Marco-Ramell et al. 2018; Housley et al. 2018), and the strategy integrated knowledge-based metabolic predictions of biotransformation routes established in-house. The possible fragments of the inferred structures of DPHP biomarker candidates can be predicted using Mass Frontier software (HighChem, Thermo Fisher Scientific, USA), which delivers small-molecule structural clarification for metabolism and metabolomics research, and then be additionally verified by the MS/MS spectra of the DPHP exposure marker candidates.

Applications of DPHP Exposure Markers in Biospecimens

The DPHP exposure marker candidates were further applied in 24H-urine samples collected from human subjects aged 17–79 and hair samples collected from long-term DPHP administrated rats. The urine sample can represent the internal exposure dose for recent DPHP exposure and the hair sample for long-term DPHP exposure.

DPHP Exposure Markers in the Human Urine Sample

A previous study reported by Hsu et al. (2019) has demonstrated the overall strategy for DPHP exposure marker discovery that integrates the HRMS-based metabolomics approach under the OPLS-DA method with the dose-response verification method in laboratory animals and finally applied the exposure marker candidates in human subjects. The overall strategy includes screening biomarkers by multivariate data analysis, verifying dose-response relationships (laboratory animals), and applying the filtered exposure marker candidates in human subjects is shown in Fig. 6 (Hsu et al. 2019). In stage I, Hsu and colleagues performed two groups of Wistar rats treated with two levels of DPHP (300 and 1200 mg/kg body weight (bw) in corn oil; $n = 15$ for each group) by oral administration to identify the DPHP exposure marker candidates (Fig. 6). Subsequent 24-h rat urine samples were collected and analyzed using LC-HRMS in full-scan mode followed by LC-MS/MS. After making the raw data of LC-HRMS into a peak list table by Progenesis QI and examining a multivariate statistical analysis by importing into OPLS-DA (the selection criteria P(corr)

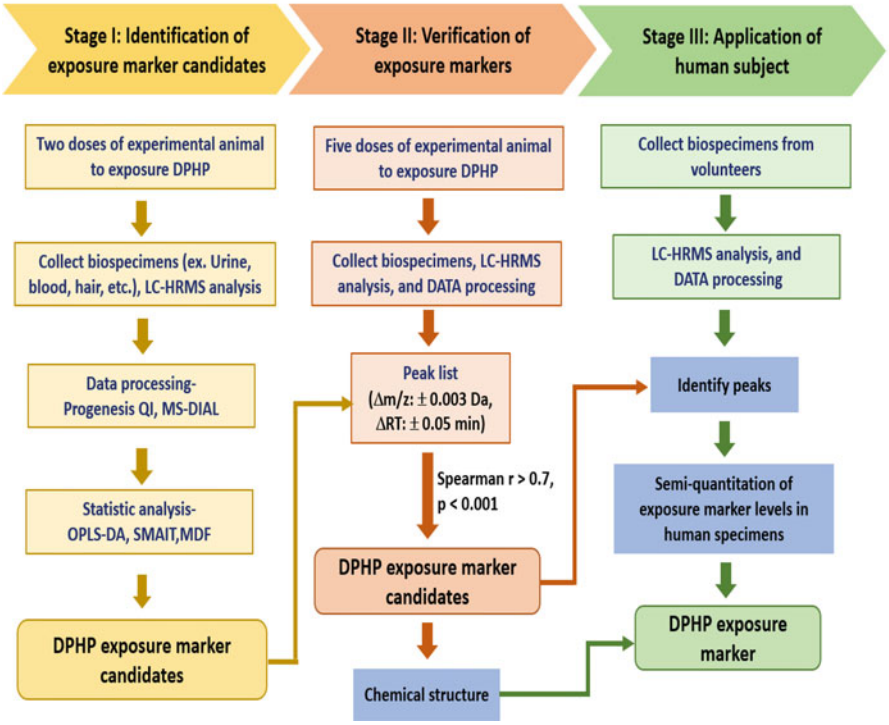


Fig. 6 Scheme of DPHP exposure marker discovery. There are three stages of DPHP exposure marker discovery, including identification of DPHP exposure marker candidates (stage I), verification of DPHP exposure marker candidates (stage II), and application in human subjects (stage III). (From the literature of Hsu et al. (2019))

[1] > 0.25 and P[1] > 5), 39 DPHP exposure marker candidates were identified (Fig. 3). Next, five groups ($n = 6$) of Wistar rats were treated with different doses of DPHP (0, 150, 300, 600, and 1200 mg/kg bw in corn oil) by oral administration to verify DPHP exposure marker candidates in stage II (Fig. 6). Thirty-six DPHP exposure marker candidates were further determined by verifying dose-response relationships for 39 DPHP exposure marker candidates. In stage III, the 36 DPHP exposure marker candidates were then investigated in 116 urine specimens from human subjects aged 17–79 years old by LC-HRMS. Twelve of the 36 DPHP exposure marker candidate signals were detected in over 30% of the human urine specimens. According to the chemical information, these 12 DPHP exposure marker signals can be divided into 7 chemicals and their corresponding isomers with the same m/z but different retention times, and 5 possible chemical structures were identified.

Three of these five possible chemical structures were oxidized the fatty acids (hydroxycapric acid [Peak 1 and the isomer P2 and P3], sebacic acid [P4 and the isomer P5], and hydroxysebacic acid [P10]) that were the typical urinary acids

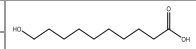
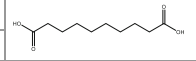
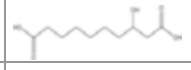
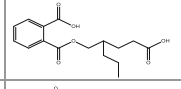
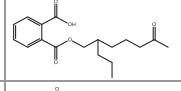
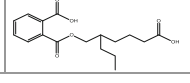
in the human body and not the specific biotransformation products of DPHP (Table 1). The other two possible chemical structures containing an aromatic dicarboxylic acid, the basic structure of phthalate, could be the specific biotransformation products of DPHP. Among them, mono-(2-propyl-4-carboxybutyl) phthalate (cx-MPBP, the P15 in Table 1) was detected in 99% of the human urine samples reported in the previous reports, but the m/z value of cx-MPBP (exact mass 307.1187) is the same with mono-(2-ethyl-5-carboxypentyl) phthalate (cx-MEPP, m/z 307.1187) that is a DEHP biotransformation product (Koch et al. 2005). The same mass of cx-MEPP and cx-MPBP cannot be distinguished through the mass spectrometer, but they can be resolved by chromatography or monitoring difference mass transitions (precursor ion/product ion). This characteristic renders cx-MPBP to be a nonspecific exposure marker for DPHP. In addition, cx-MEPP can be an appropriate DPHP exposure marker due to the extensive distributed cx-MEPP levels in the human research subjects if the isomers (cx-MEPP and cx-MPBP) can be discriminated by chromatographic methods and measured in a single LC-MS/MS run. Mono-(2-propyl-7-dihydroxyheptyl)-phthalate (di-OH-MPHP, P25 and the isomer P26 in Table 1) contains a complete side chain derived from DPHP and shows the high detection rates (96%, 111/116 and 74%, 86/116, respectively) and the maximum signal abundance among the 36 exposure marker candidate signals (Table 1). Thus, di-OH-MPHP is thought over to be a DPHP specific exposure marker.

DPHP Exposure Markers in Hair Samples

Hair has been increasingly used as a suitable matrix to assess the exposure of short- and long-term exposure reflection because chemicals found in serum can migrate into hair (Wennig 2000; Covaci et al. 2002; Alves et al. 2014). Shih and colleagues used the urine and hair samples from experimental animals to clarify whether the exposure markers in urine can be applied in hair samples as the long-term exposure marker (Shih et al. 2019a). The urine and hair sampled were collected from Wistar rats treated with five different doses of DPHP (0, 150, 300, 600, and 1200 mg/kg bw in corn oil) by oral administration once every 24 h for 7 days, in which the urine samples were collected on day 1, 7, 14, and 28 after the first exposure of DPHP, as well as the hair samples, were collected on day 28. The raw MS data were converted to peak lists using Progenesis QI software and identify metabolite candidates by OPLS-DA (the selection criteria P(corr) [1] > 0.5 and P[1] > 0.1 for urine samples, whereas P(corr) [1] > 0.5 and P[1] > 0.01 for hair samples).

The number of signals shows a dose-dependent response in the urine samples on days 1, 7, 14, and 28, and the hair samples on day 28 were 37, 43, 31, 7, and 29 candidates, respectively. After 7 continuous days of exposure, the number of DPHP metabolites present in urine is higher than the amount after 1 day of exposure. However, more tentative DPHP metabolites can be identified in urine samples on day 7 (43 candidates) than in hair samples on day 28 (29 candidates), and only 10 DPHP metabolites in the urine samples were also found in the hair samples,

Table 1 Characteristics of validated DPHP exposure markers in urine samples from humans and rats. (From the literature of Hsu et al. (2019), Shih et al. (2018, 2019a, b)

Peak no.	m/z for biotransformation product signals	RT (min)	Markers verified by rat	Literature	Chemicals information	
					Structure	Name
P1	187.1346	3.57	+			Hydroxycapric acid
P2	187.1346	3.67	+			
P3	187.1346	3.75	+			
P4	201.1138	2.29	+			Sebacic acid
P5	201.1138	2.59	+			
P6	203.1294	2.84	+			
P7	203.1295	2.41	+			
P8	203.1295	2.95	+			
P9	215.1296	3.22	+			
P10	217.1088	1.93	+			Hydroxysebacic acid
P11	228.1613	3.45	+			
P12	253.1124	2.38	+			
P13	292.1234	2.45	–			
P14	300.1827	3.34	+			
P15	307.1196	2.98	+			Mono-(2-propyl-4-carboxybutyl) phthalate (cx-MPBP)
P16	319.1563	3.53	+			Mono-(2-propyl-6-oxoheptyl phthalate
P17	321.1352	3.31	+			Mono-(2-propyl-5-carboxypenyl) phthalate

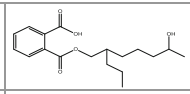
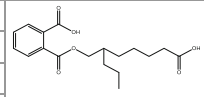
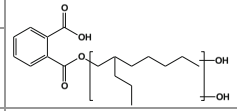
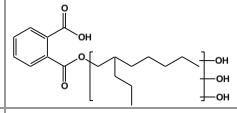
P18	321.1719	3.84	+			Mono-(2-propyl-6-hydroxyheptyl) phthalate
P19	323.1146	2.13	+			
P20	335.1509	3.57	+			Mono-(2-propyl-6-carboxyhexyl) phthalate (cx-MPHxP)
P21	335.1509	3.68	+			
P22	335.1511	3.93	+			
P23	335.1511	3.06	+			
P24	337.1308	2.78	+			
P25	337.1664	2.52	+			Mono-(2-propyl-dihydroxyheptyl) phthalate (Di-OH-MPHP)
P26	337.1664	2.89	+			
P27	351.1454	2.43	+			
P28	351.1456	2.95	+			
P29	351.1456	3.09	+			
P30	353.1613	2.14	+			Mono-(2-propyl-trihydroxyheptyl) phthalate (Tri-OH-MPHP)
P31	361.1509	2.52	–			
P32	363.1459	2.69	+			
P33	365.1615	3.21	+			
P34	367.1405	2.08	+			
P35	367.1405	2.15	+			
P36	499.2198	2.27	+			
P37	511.1833	2.18	–			
P38	513.1989	2.27	+			
P39	513.1989	2.63	+			

Table information are from the literature of Hsu et al. (2019), Shih et al. (2018, 2019a, b)

proving that the DPHP metabolites in urine and hair are different. To clarify why the metabolites were different between urine and the 28th-day hair samples, the chemical structures of the identified metabolites were determined by UPLC-MS/MS. The structures of tentative metabolites in the 7th-day urine and 28th-day hair samples were identified. These metabolites seem to be structurally related to DPHP, including three known DPHP metabolites (oxo-MPHP, OH-MPHP, and cx-MPHxP), one previously reported DPHP structure-related metabolite that has been suggested as a suitable DPHP exposure marker (P25, di-OH-MPHP) searched/filtered by MDF and SMAIT (Shih et al. 2018), and three groups of novel DPHP structure-related metabolites (m/z = 321.1358, 337.1670, and 337.1671, respectively) (Shih et al. 2019a). These DPHP-related signals showed a dose-dependent response in urine samples but no dose-dependent response in hair samples. Among the urine exposure markers, only the cx-MPHxP signal can be detected in the first visit samples. These results indicate that the hair samples can be long-term exposure samples and use different exposure markers than urine samples.

Mini-dictionary of Terms

- ***Biotransformation products*** are metabolized from an exogenous compound to increase the water solubility and excretion efficiency.
- ***HRMS*** is a high-resolution mass spectrometer that can provide accurate mass measurement and comprehensive recording of the signal.
- ***Metabolomics*** is a scientific research of chemical processes involving metabolites, small molecule substrates, intermediates, and products of cell metabolism and providing a direct functional readout of the physiological state in organisms.
- ***Dose-response*** is that an increasing level of exposure is related to either an increasing or a decreasing risk of the outcome.
- ***MS/MS*** is a particular m/z value of interest selected from the mass spectrum and collided into fragment ions to identify the chemical structure.
- ***Multiple reaction monitoring (MRM)*** is a precise and sensitive mass spectrometry technique to selectively quantify compounds within complex mixtures.

Key Facts of DPHP Exposure Markers

DPHP is widely used in personal care products and plastic products in recent years. Due to the subchronic toxicity of DPHD, it is necessary to biomonitor and estimates the DPHP exposure in the human body. DPHP has short half-lives in the human body and exhibits rapid ADME (absorption, distribution, metabolism, and excretion) processes within a day. Biomonitoring of DPHP exposure is commonly performed by measuring its respective metabolites instead of their original chemical forms. Discriminant the DPHP metabolic features and identification of DPHP exposure markers are essential to discover the exposure markers.

Summary Points

- This chapter shows an HRMS-based metabolomics profiling to screen the DPHP exposure markers.
- The DPHP exposure markers are identified and verified in experimental animals and then be applied to human subjects.
- Three metabolomics data-screening approaches, OPLS-DA, SMAIT, and MDF, were introduced in this chapter to discover the exposure marker candidates.
- The biotransformation product of DPHP (oxo-MPHP, OH-MPHP, cx-MPHxP, and di-OH-MPHP) can be the exposure marker for general human exposure assessments in urine samples.

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Biomarkers of Neurotoxicity

15

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Contents

Abstract

Many data indicate that long-term exposure to an increasing number of neurotoxic xenobiotics in the environment plays an important role in the pathogenesis of neurodegenerative diseases. In fact, a correlation between exposure to some

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xenobiotics and the risk of developing these diseases has been demonstrated in epidemiological studies. The mechanisms of the neurotoxic action of individual xenobiotics are not yet fully understood, but the main mechanisms leading to damage to nerve cells by xenobiotics are similar to those shown in neurodegenerative diseases. In this chapter, we present current data on the main mechanisms of the neurotoxic effects of selected xenobiotics, including the induction of oxidative stress, neuroinflammation, disrupting metabolic processes, and inducing excitotoxicity and, as a result, intensifying cell death. We also present the potential role of these xenobiotics in the pathogenesis of neurodegenerative diseases, the possibility of estimating exposure to them, and the available methods of assessing the effects of their damaging effects on nerve cells, including biomarkers of oxidation by free radicals, markers of microglia activation, cytokine profiling, level of S100β protein and prolactin, and imaging studies.

Keywords

Oxidative stress · Neuroinflammation · Metabolic disturbances · Excitotoxicity · Cell death · Neurotoxicity · Xenobiotics · Free radicals · Microglia activation · Glutamate · Heavy metals · Pesticides

Abbreviations

4-HNE	4-hydroxy-2-nonenal
8-OHdG	7,8-dihydro-8-oxodeoksyguanosine
8-OHG	7,8-dihydro-8-oxoguanosine
AA	Arachidonic acid
ACD	Accidental cell death
AD	Alzheimer's disease
AGEs	Advanced glycosylated end products
ALS	Amyotrophic lateral sclerosis
Apaf-1	Apoptotic protease-activating factor-1
AT	Atrazine
Bad	Bcl-2-associated agonist of cell death
Bak	Bcl-2 antagonist/killer 1
Bax	Bcl-2-associated X protein
BBB	Blood-brain barrier
Bcl-2	B-cell lymphoma-2
Bid	BH3-interacting domain death agonist
Bim	Bcl-2-like protein 11
BP-3	Benzophenone-3
CAT	Catalase
CB2R	Cannabinoid receptor 2
CD	Cluster of differentiation
CNS	Central nervous system
CSF	Cerebrospinal fluid
CSF1R	Colony-stimulating factor 1 receptor

CX3CR1	Gai-coupled 7-transmembrane chemokine receptor
DHA	Docosahexaenoic acid
DLP1	Dynamin-like protein 1
EAAT1–5	Excitatory amino acid transporters
EDCs	Endocrine-disrupting compounds
ETC	Electron transport chain
F2-IsoPs	F2-isoprostanes
F4-NeuroPs	F4-neuroprostanes
FACS	Fluorescence-activated cell sorting
FADD	Fas-associated death domain
FRs	Free radicals
GBH	Glyphosate-based herbicides
GC/NICI-MS	Gas chromatography/negative-ion chemical ionization mass spectrometry
Glu	Glutamate
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GST	Glutathione transferase
HD	Huntington's disease
HPLC	High-performance liquid chromatography
Iba-1	Ionized calcium-binding adaptor molecule 1
IL	Interleukin
IsoFs	Isofurans
LC/MS/MS	Liquid chromatography/tandem mass spectrometry
LC-EC	Liquid chromatography with electrochemical detection
MDA	Malondialdehyde
MDMA	3,4-methylenedioxymethamphetamine
Mfn2	Mitofusin 2
MIP-1α	Macrophage inflammatory protein-1α
MMP	Matrix metalloproteinase
MOMP	Permeabilization of the outer mitochondrial membrane
MPT	Mitochondrial permeability transition
mtPTP	Mitochondrial transition pore
MZ	Mancozeb
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate ($NADP^+$)
NeuroFs	Neurofurans
NF-κB	Nuclear factor kappa B
NLRP3	Nod-like receptor family protein 3
NMDAR	N-methyl-D-aspartate receptor
NoxA	Phorbol-12-myristate-13-acetate-induced protein 1
NPs	Nanoparticles
NRF-1	Nuclear respiratory factor 1
NRF-2	Nuclear factor erythroid 2-related factor 2

OS	Oxidative stress
P2X7R	P2X ligand-gated ion channel type 7 receptor
P2Y12R	Purinergic metabotropic 12 receptor
PD	Parkinson's disease
PET	Positron emission tomography
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
P-gp	P-glycoprotein
PM 2.5	Particulate matter
PRRs	Pattern recognition receptors
PUMA	p53 upregulated modulator of apoptosis
RAGE	Receptor for advanced glycation end products
RCD	Regulated cell death
ROS	Reactive oxygen species
rt-PCR	Real-time polymerase chain reaction
S100β	S100 calcium-binding protein β
sICAM	Soluble intracellular cell adhesion molecules
SOD	Superoxide dismutase
SPECT	Single-photon emission computed tomography
sVCAM	Soluble vascular cell adhesion molecules
TBA	Thiobarbituric acid
TBT	Tributyltin
TFAM	Mitochondrial transcription factor A
TLRs	Toll-like receptors
TNF-α	tumor necrosis factor alpha
Trx	Thioredoxin
TSPO	Translocator protein
Tyr-NO_2	Nitrotyrosine
VDCC	Voltage-dependent calcium channels
xc^-	Cystine/glutamate antiporter
ZnONPs	Zinc oxide nanoparticles

Introduction

A lot of data show that increasing incidence of neurodegenerative diseases is associated not only with the extension of human life but also to a large extent with environmental pollution. The main sources of environmental pollution caused by human activity are industrial waste, automobile exhaust, compounds formed during waste incineration, intensive agriculture practices especially ungoverned manufacturing, and wrong agriculture practices (Iqubal et al. 2020). Most of the data concerns the neurotoxic effects of heavy metals (e.g., lead, arsenic, cadmium, mercury), pesticides (e.g., rotenone, paraquat, glyphosate), and endocrine-disrupting compounds (EDCs) such as polychlorinated biphenyls, polycyclic aromatic hydrocarbons, bisphenol A, and UV filters. Several epidemiological studies showed an

association between occupational exposure to some xenobiotics and the prevalence of neurodegenerative diseases. For example, a link has been shown between exposure to organophosphate and carbamate pesticides, fumigants, defoliants, and Alzheimer's disease (AD) and between Parkinson's disease (PD) and exposure to pesticides (Tyas et al. 2001; Brown et al. 2005). However, epidemiological studies on the relationship between exposure to heavy metals or solvents and development of neurodegenerative diseases are inconclusive, because not all studies show such correlation. A lot of preclinical data indicates neurotoxic effects of many environmental pollutants, especially lipophilic or small chemical compounds. Moreover, because lipophilic xenobiotics can cross the placenta and the blood-brain barrier (BBB) is more permeable at this stage of life, these compounds can evoke developmental neurotoxicity or increase the brain's susceptibility to damage induced later in life. Both the mechanisms of the neurotoxic action of individual xenobiotics and the etiology of neurodegenerative diseases are not fully understood yet; however, the main mechanisms leading to damage to nerve cells, especially the most sensitive neurons, are similar in neurodegenerative diseases to these caused by some xenobiotics. And although many factors (genetic, epigenetic, age, psychological stress, comorbidities, hormonal disorders) are involved in the pathogenesis of neurodegenerative diseases, it has also been shown that long-term exposure to the neurotoxic xenobiotics present in the environment induces or significantly accelerates the negative age-related changes in the morphology and function of nerve cells. Nerve cells are particularly susceptible to damage because they contain poor antioxidant protection, and as a result of intensive metabolism, mainly oxidative phosphorylation and the transformation of certain neurotransmitters, primarily dopamine, they produce a much amount of free radicals (FRs). Oxidative stress (OS) results in lipid peroxidation; damage to proteins, nucleic acids, and carbohydrates; disruption of Ca^{2+} homeostasis; reduction of ATP levels; and, consequently, dysfunction and subsequent cell death. As clinical symptoms of neurodegenerative diseases are most often diagnosed many years after the onset of neurodegenerative changes, so, it would be important to develop a reliable marker of early neurodegenerative changes. In the case of neurotoxic xenobiotics, it is possible to assess exposure to them by determining the level of parent compounds or metabolites in blood or urine; however, their neurotoxic effects in humans are not yet routinely assessed in imaging and functional studies. In this chapter, we present the current data on the main mechanisms of the neurotoxic action of selected xenobiotics (Fig. 1), their potential role in the pathogenesis of neurodegenerative diseases, and the possibility of estimating exposure to them and assessing their neurotoxic effects.

Biomarkers of Oxidative Stress

Oxidative Stress: Definition, Causes, and Effects

Oxidative stress (OS) is defined as an imbalance in a redox system, which may arise due to increased production of free radicals (FRs), mainly reactive oxygen species (ROS) and reactive nitrogen species (RNS), or impairment of antioxidant ability in

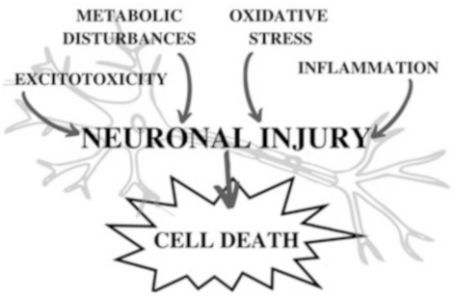

Fig. 1 The main mechanisms of the neurotoxic action of xenobiotics

Fig. 2 General concept of oxidative stress that is demonstrated as the imbalance between generation of free radicals and antioxidant defense

biological system (Fig. 2). ROS and RNS produced in physiological amounts play the role of mediators and regulators, ensuring the proper functioning of cells. However, high concentration and long-time exposure of the high reactive FRs may damage cellular components (carbohydrates, proteins, lipids, DNA), which consequently may lead to cell death (Valko et al. 2007).

FRs arise as a result of naturally occurring metabolic processes in the cells, such as aerobic respiration in the mitochondrial electron transport chain (ETC). Moreover, many exogenous factors can accelerate their formation and consequently constitute an additional, relevant source of the production of a significant amount of FRs, e.g., stress, improper diet, smoking, alcohol consumption, infections, pollution, and ionizing radiation. Numerous FRs are also produced in the metabolism of xenobiotics as a result of natural detoxification processes in the organism. Among these xenobiotics, especially heavy metals, pesticides and solvents can be distinguished. Evaluation of potential occurrence of OS is also necessary in discovery and development of new drugs, especially used in central nervous system (CNS) diseases.

Due to the high consumption of oxygen and high content of lipids, CNS is particularly vulnerable to damage caused by OS. Moreover, OS in CNS may contribute to activation of other mechanisms of neurotoxicity, i.e., glutamate-induced excitotoxicity mechanism and hyperstimulation of N-methyl-D-aspartate receptors (NMDAR) (Siwek et al. 2013).

Despite that the ethology of the many disorders in the CNS is still largely unknown, recent studies have shown that OS may play an important role in the development of neurodegenerative diseases, for instance, PD, AD, Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) (Niedzielska et al. 2016), as well as psychiatric disorders (e.g., depression, bipolar disorder, schizophrenia) (Siwek et al. 2013).

Biomarkers

FRs have extremely short half-lives, so it is difficult to measure them in vivo directly. Therefore, evaluation of OS is based on measurement of biomarkers in biological fluids and/or tissues, which indirectly correlate with damage caused by oxidation, i.e., specific products of lipid, protein, carbohydrates, and DNA oxidation. Figure 3 illustrates the main products of oxidation by FRs, which are described in the text below. Additional information on exposure to OS is provided by the determination of the activity of antioxidant enzymes and the level of nonenzymatic antioxidants.

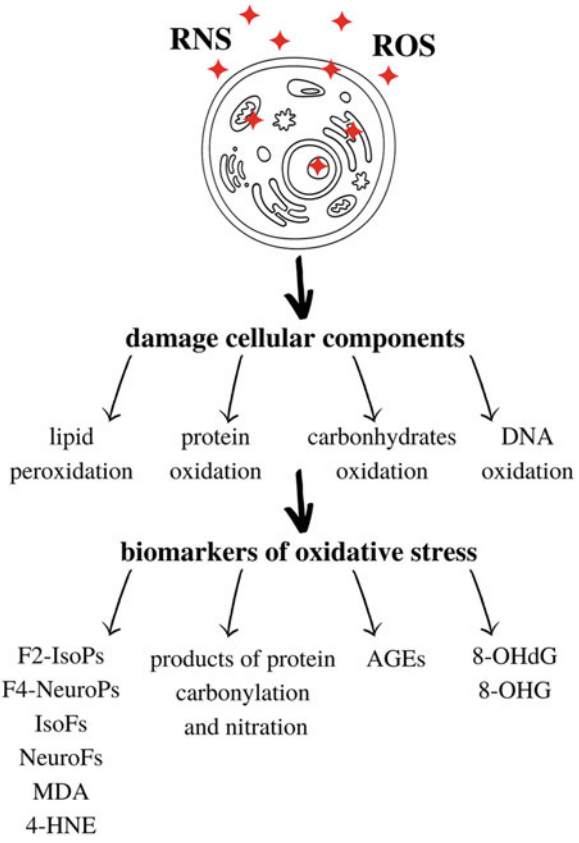

Fig. 3 Effects of oxidative stress on a molecular level and main products of oxidation cellular molecules. 4-HNE, 4-hydroxy-2-nonenal; 8-OHdG, 8-hydroxydeoxyguanosine; 8-OHG, 8-hydroxyguanosine; AGEs, advanced glycosylated end products; F2-IsoPs, F2-isoprostanes; F4-NeuroPs, F4-neuroprostanes; IsoFs, isofurans; MDA, malondialdehyde; NeuroFs, neurofurans; RNS, reactive nitrogen species; ROS, reactive oxygen species

In this subsection, we present a list of OS biomarkers that can be determined in in vitro and in in vivo tests in biological fluids such as urine, serum, plasma, and cerebrospinal fluid (CSF). It needs to be highlighted that most of these markers are not very specific to the CNS because observed changes may be associated with the toxic effects of xenobiotics on other organs. However, determination of the level of these markers in vivo, especially when combined with other neurotoxicity studies, may be important in assessing the effect of xenobiotics and drugs on the CNS and in determining the pathomechanism of neurodegenerative diseases.

Products of Lipid Peroxidation

F2-Isoprostanes (F2-IsoPs) and F4-neuroprostanes (F4-NeuroPs)

F2-IsoPs are a group of compounds produced via nonenzymatic peroxidation of arachidonic acid (AA), which are widely considered as the "gold standard" for evaluation of OS in vivo.

On the other hand, F4-NeuroPs belong to a group of IsoP-like compounds, termed neuroprostanes, formed by the oxidation of docosahexaenoic acid (DHA), the main component of neuronal membranes. Therefore, in contrast to F2-IsoPs, F4-NeuroPs are considered as the only quantitative in vivo biomarker of damage induced by OS that is selective for neurons.

Detection of F2-IsoPs and F4-NeuroPs in human body fluids, using gas chromatography/negative-ion chemical ionization mass spectrometry (GC/NICI-MS), is considered the most sensitive and robust method (Yen et al. 2015).

Isofurans (IsoFs) and neurofurans (NeuroFs)

IsoFs and NeuroFs are products of peroxidation of AA and DHA, respectively. These compounds are formed under conditions of increasing oxygen concentration. Therefore, they can be used as biomarkers of oxidative injury in condition of high oxygen tension. However, analysis of NeuroPs, like F4-NeuroFs, may be particularly important in assessing the effect of OS on lipid peroxidation in the brain. Currently, the preferred method of determination of IsoFs and NeuroFs is liquid chromatography/tandem mass spectrometry (LC/MS/MS) (Song et al. 2008).

Malondialdehyde (MDA)

MDA is high reactive and stable product of lipid peroxidation of membrane omega-3 and omega-6 fatty acids. Additionally, MDA is also formed as a by-product of thromboxane and prostaglandin biosynthesis, and studies have shown that this compound may regulate gene expression and play a role as signaling messenger.

A widely used and relatively easy to perform assay used to detect MDA is the test based on the reaction of MDA with thiobarbituric acid (TBA). However, due to low specificity of this reaction, high-performance liquid chromatography (HPLC) is currently a recommended method for detection of MDA in biological samples.

4-Hydroxy-2-nonenal (4-HNE)

4-HNE is an α,β-unsaturated aldehyde neurotoxic product of lipid peroxidation. 4-HNE is considered as a "second messenger of free radicals," which forms adducts with proteins and acts as a signaling factor. Additionally, the presence of protein adducts can be also used as biomarkers of OS.

Direct detection of 4-HNE may be insensitive and complicated; therefore, immunoassays (i.e., immunohistochemistry and 4-HNE-His ELISA), which use specific monoclonal antibodies against HNE-histidine epitope, are currently widely applied (Borovic et al. 2006).

Products of Nucleic Acid Oxidation

8-Hydroxydeoxyguanosine (7,8-dihydro-8-oxodeoksyguanosine, 8-OHdG) and 8-hydroxyguanosine (7,8-dihydro-8-oxoguanosine, 8-OHG)

OS induce oxidation of DNA and RNA resulting in an uprising 8-hydroxydeoxyguanosine (7,8-dihydro-8-oxodeoksyguanosine, 8-OHdG) and 8-hydroxyguanosine (7,8-dihydro-8-oxoguanosine, 8-OHG), which are well-characterized products of DNA and RNA oxidation, respectively. Both products are excreted in the urine, and their determination in this biological fluid may provide information about the presence of OS in the body. Therefore, their use in the assessment of OS in a specific tissue is limited. However, studies show that measuring 8-OHG may correlate with the development of neurodegenerative diseases (Nunomura et al. 2012). In addition, both nucleic acid oxidation products can be determined in the CSF.

The preferred and most reliable technique for the determination of 8-OHdG and 8-OHG is LC/MS/MS. Moreover, liquid chromatography with electrochemical detection (LC-EC) is also acceptable. However, analyses using immunological methods are not recommended (Weimann et al. 2012).

Other Products of Oxidation

Protein Carbonyl and Protein Nitration

Carbonylation and nitration are common protein modifications that take place as consequence of severe OS. In humans, increased levels of elevated protein carbonyl have been reported in numerous conditions, including neurodegenerative diseases (Greilberger et al. 2008). Also, product of protein nitration, nitrotyrosine (Tyr-NO_2), is considered as useful biomarker of OS, especially in disorders with inflammation.

Advanced Glycosylated End Products (AGEs)

AGEs are compounds that are formed by nonenzymatic reactions between reducing sugars and proteins, lipids, or nucleic acids. The formation of these harmful products

is also a consequence of OS. On the other hand, AGEs may increase OS, which may lead to an exacerbation of their toxic effect. Moreover, AGEs are responsible for the induction of inflammation (Miranda and Outeiro 2010).

Biomarkers of Antioxidant Defense

As a result of exposure to neurotoxic xenobiotics, it may not only increase the production of FRs but also weaken the antioxidant capacity. Therefore, evaluations of enzymatic and nonenzymatic compounds of antioxidant defense are also applied. The crucial antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione transferase (GST). Among nonenzymatic antioxidants are mainly glutathione (GSH), vitamins (A, E, C), thioredoxin (Trx), flavonoids, trace elements, and proteins, i.e., albumin, ceruloplasmin, and metallothionein (Niedzielska et al. 2016).

Neuroinflammation and Xenobiotics

One of the main mechanisms of toxic action of selected xenobiotics on the CNS is neuroinflammation. It is a complex inflammatory response to various damaging triggers, e.g., trauma, autoimmunity, stroke, or toxin. This physiological response is mediated by cytokines, ROS, and other inflammatory factors, which are produced by all kind of cells residing in the CNS, including neurons and oligodendrocytes but predominantly microglia and astrocytes as well as endothelial cells and recruited peripherally derived immune cells (DiSabato et al. 2016; Shabab et al. 2017). Although the aim of neuroinflammation is to maintain homeostasis through the reinforcement of the CNS immunity in response to potentially damaging stimuli, it can have both beneficial and adverse effect. Especially, chronic or exaggerated neuroinflammatory response gets out of control, resulting in dysregulated overproduction of proinflammatory cytokines and involving adaptive immunity. As a consequence, such state leads to impaired synaptic plasticity, OS, metabolic disturbances due to the mitochondrial dysfunction and the disruption of the BBB. It is particularly noteworthy that all of these processes may trigger neurodegeneration (Werry et al. 2019). Figure 4 presents the main mechanisms of neuroinflammation described below.

The pivotal moment in neuroinflammation is microglial activation. These resident brain macrophages round up, getting amoeba-like shape; proliferate; and migrate following activation; then, they can present antigens to T-cells and phagocyte and release a wide variety of potentially neurotoxic compounds like proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) or interleukin 1 (IL-1) (Shabab et al. 2017). Markers of microglia activation are frequently used in animal studies of xenobiotic action; for example, in research on benzophenone-3 (BP-3) mechanism of action, ionized calcium-binding adaptor molecule 1 (Iba-1), which is a sensitive marker for microglia as well as cluster of differentiation (CD) 40, a

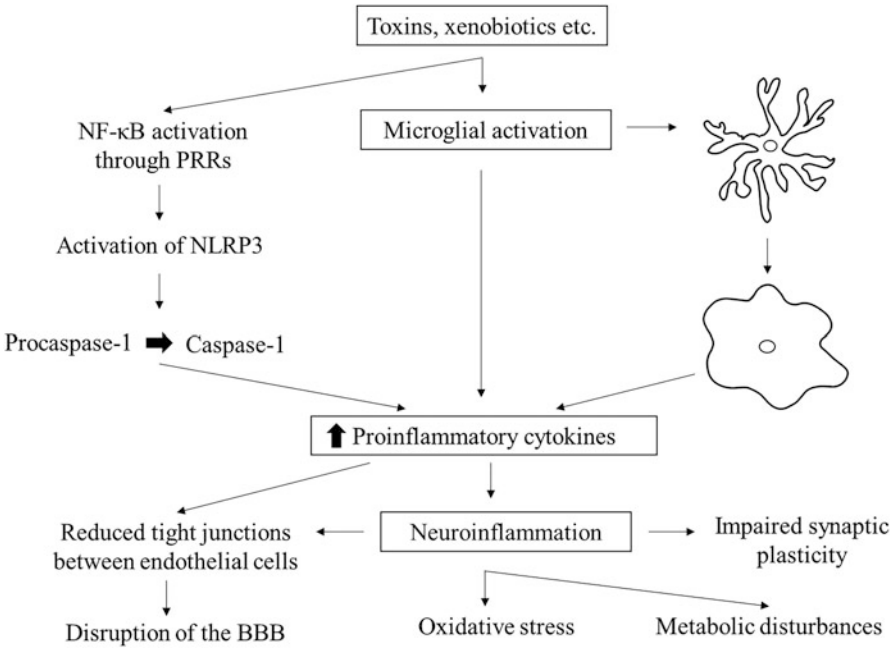

Fig. 4 The main mechanisms of neuroinflammation

costimulatory receptor occurring on the antigen-presenting cells, and C1q, an initiating protein in the classical pathway of complement activation, were investigated in selected rat brain structures in both mRNA and protein levels using real-time polymerase chain reaction (rt-PCR), Western blot, and immunochemistry techniques (Pomierny et al. 2019). Microglial polarization, likewise, can be analyzed through the expression of CD86 – associated with cytotoxic, classically activated M1-like microglia – and CD206, cytoprotective, alternatively activated M2 cells (Fig. 5). Usually, this kind of study is conducted using more automated methods such as fluorescence-activated cell sorting (FACS) (Hellström Erkenstam et al. 2016). What is more, the level of pro−/anti-inflammatory cytokines can be investigated; for example, in rats exposed to diesel exhaust by inhalation, the increased level of TNF-α, IL-1β, IL-6, and chemokine macrophage inflammatory protein-1α (MIP-1α) was determined using ELISA (Levesque et al. 2011). Novel multiplex technologies for cytokine profiling are also used in both research and clinical settings (Chowdhury et al. 2009).

The complex cascade of processes in neuroinflammation includes activation of nuclear factor kappa B (NF-κB) – proinflammatory transcription factor through toll-like receptors (TLRs; belonging to the family of pattern recognition receptors (PRRs)), mainly TLR4. This is one of the main mechanisms underlying metals' (also contained in polluted air) as well as ethanol's toxicity (Ibáñez et al. 2019; Bondy 2021). Furthermore, these xenobiotics elevate the level of other

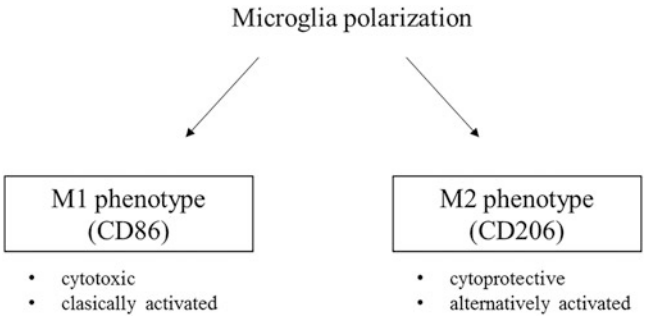

Fig. 5 Microglial polarization. CD – cluster of differentiation

inflammatory-related proteins, especially Nod-like receptor family protein 3 (NLRP3) inflammasome – composed of the NLRP3 protein, procaspase-1, and the adaptor protein. Inflammasomes are large multi-protein complexes localized in the cytoplasm that contain PRRs. Their activation upon cellular stress may be a common mechanism that connects the exposure to toxicants like heavy metals and pesticides with neuroinflammation and neurodegenerative disorders. Activated NLRP3 leads to the cleavage of procaspase-1 that promotes the secretion of pro-inflammatory cytokines IL-1β and IL-18 (Wang et al. 2013; Anderson et al. 2018).

Proinflammatory cytokines are known to elevate permeability of the BBB by reducing tight junctions between brain endothelial cells. The soluble form of the membrane adhesion molecule CD146, primarily expressed at the intracellular junctions of endothelial cells, was found to be increased in CSF of patients with neuroinflammatory diseases. This elevation strongly correlated with CSF neuroinflammation-related factors, such as matrix metalloproteinases (MMP2, MMP9), soluble adhesion molecules (intracellular and vascular cell adhesion molecules – sICAM and sVCAM), cytokines (i.e., TNF-α, IL-1β, IL-10), and clinical parameters, making this molecule a promising marker for future studies. Moreover, in in vitro model of the BBB sCD146 significantly decreased tight junctions of protein expression and promoted the apoptosis (programmed cell death, described in detail in the following subsections) of hCMEC/D3 cells (Wang et al. 2020).

On the one hand, in principle, activated microglia leads to remove pathogens, but on the other hand, it may contribute to neurodegeneration being the cause of neuronal dysfunction and death (Shabab et al. 2017). As it has been already mentioned in previous subsections, a growing body of evidence links the exposure to neurotoxic xenobiotics with the development of neurodegenerative diseases. Indeed, neuroinflammation is not only a common effect of xenobiotic action but also frequently observed feature of a wide variety of CNS diseases, including psychiatric disorders, stroke, and neurodegenerative diseases (Werry et al. 2019). Therefore, noninvasive techniques for imaging neuroinflammation are the most preferred ones in animal and clinical studies, allowing early diagnosis of the disease and real-time monitoring of neuroimmune responses, i.e., during selected therapy. Positron emission tomography (PET) is a widely used technique based on a

radiolabeled molecule characterized by the ability to cross the BBB as well as high affinity and selectivity in tandem with low nonspecific binding (Jain et al. 2020). To date, a lot of neuroinflammation markers – molecular targets for radiotracers – have been described, for example, receptor for advanced glycation end products (RAGE), Gai-coupled 7-transmembrane chemokine receptor (CX3CR1), P2X ligand-gated ion channel type 7 receptor (P2X7R), purinergic metabotropic 12 receptor (P2Y12R), colony-stimulating factor 1 receptor (CSF1R), cannabinoid receptor 2 (CB2R), cyclooxygenase-1, and cyclooxygenase-2, but the most popular is translocator protein (TSPO) (Narayanaswami et al. 2018; Jain et al. 2020). It is noteworthy that selected molecular targets may perform various functions besides being up-/downregulated in neuroinflammatory processes. TSPO is 18 kDa outer mitochondrial membrane protein upregulated in neuroinflammation. Even though TSPO is the most widely used imaging biomarker of neuroinflammation, it does have some limitations. Above all, there is no certainty whether the presence of this marker in PET imaging indicates favorable or toxic inflammatory responses. Moreover, a nucleotide polymorphism frequently leads to low ligand affinity. It indicates the need to continuous improvement of methodology and to develop subsequent novel generations of radiotracers (Werry et al. 2019).

The Role of Metabolic Disturbances in the Neurotoxic Effects of Xenobiotics

Brain cells, mainly neurons, need a large amount of energy in the form of ATP to ensure their proper function, especially to maintain normal neuronal excitability, the process of neurotransmission and formation of NADPH required for the removal of FRs. The main source of energy in the brain is glucose, and the first stage of metabolism, the process of glycolysis, takes place primarily in astrocytes, and then, part of the produced lactate is delivered to neurons (Tang et al. 2014). In turn, oxidative phosphorylation, the process that supplies the greatest amount of ATP, is mainly carried out in neurons, and its performance depends on mitochondrial function including the correct mitochondrial membrane potential, mitochondrial dynamics (fusion-fission processes), biogenesis of mitochondria, and the process of mitophagy. Mitochondria, in addition to regulate energy production, are significantly involved in calcium homeostasis, apoptotic process, and production of ROS. Current research indicates that abnormal energy metabolism often associated with mitochondrial dysfunction, by reducing the energy supply to neurons, can induce or intensify neurodegeneration processes. Mitochondrial dysfunction is also a common cause of ROS overproduction, because the significant endogenous source of ROS is the leakage of electrons from the mitochondrial ETC. Neurotoxic effects of most of the xenobiotics are related to the induction of OS, and current data indicates that this effect may result from metabolic and mitochondrial disturbances. Moreover, perturbations in Ca^{2+} homeostasis and/or ROS overproduction can induce mitochondrial apoptosis pathway (by increase of permeability of mitochondrial transition pore (mtPTP) and increase of cytochrome c release) or mitochondrial permeability

transition (MPT)-driven regulated necrosis. Many xenobiotics can disrupt metabolic processes, but most of the evidence concerns the involvement of this mechanism in the neurotoxic effects of heavy metals and pesticides. Targets of neurotoxic xenobiotic action on metabolic processes are presented in Fig. 6.

The Metabolic Disturbances Induced by Heavy Metals

It has been found that chronic exposure to arsenic leads to the weakening of oxidative phosphorylation by inhibiting complexes I, II, and IV, which results in increased ROS production, protein carbonylation, and lipid peroxidation (Prakash et al. 2015). Besides its influence on oxidative phosphorylation, arsenic also weakens the mitochondrial biogenesis process, by reduction of the levels of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and its downstream transcription factors nuclear respiratory factor 1 (NRF-1), nuclear factor erythroid 2-related factor 2 (NRF-2), and mitochondrial transcription factor A (TFAM), which leads to the induction of the apoptosis process (Prakash and Kumar 2016). Also, the administration of sodium arsenite in the prenatal and early postnatal periods causes a decrease in the activity of mitochondrial complexes (I, II, and IV), mitochondrial membrane potential, and antioxidant activity (by reducing the expression of CAT, SOD, GPx, and GSH) in the frontal cortex, hippocampus, and striatum in the rats (Chandravanshi et al. 2018). These changes are accompanied by an increase in ROS production, lipid peroxidation, and protein carbonylation; an increase in the ratio of pro-apoptotic to anti-apoptotic proteins; and activation of caspase-3, the effector enzyme of apoptosis. The neurotoxic effects of arsenic in the developmental period induce cognitive dysfunctions, including disturbances of learning and memory. Arsenic exposure is postulated to contribute to the pathogenesis of AD. Studies in rats have shown that inorganic arsenic increases the production of β-amyloid and the activity of β-secretase. In a mouse model of AD, arsenic was shown to increase the deposition of amyloid and the phosphorylated form of the Tau protein, which correlates with bioenergetic dysfunction and changes in redox metabolism (Garza-Lombó et al. 2019). In a case-control study, a correlation has been shown between an increased risk of developing AD with high urinary inorganic arsenic and lower levels of dimethylarsinic acid (Yang et al. 2018).

Mitochondrial dysfunction and OS are also the main mechanisms involved in neurotoxic effect of iron and manganese excess. Easy switch between $Fe2^{+}$ and Fe^{3+} and interactions with hydrogen peroxide in Fenton's reaction lead to production of FRs, but excess Fe also causes changes in the morphology of the mitochondria, lowers the mitochondrial membrane potential, and decreases production of ATP. There is a lot of evidence that excess iron or disruption of the factors regulating its homeostasis may be important in the pathogenesis of neurodegenerative diseases (Chen et al. 2019). Like iron, manganese, as a cofactor of numerous enzymes, is essential for brain function, but its excess has a neurotoxic effect, in which the reduction of energy metabolism plays an important role. Manganese has been shown to disrupt mitochondrial function, causing a decline in mitochondrial membrane

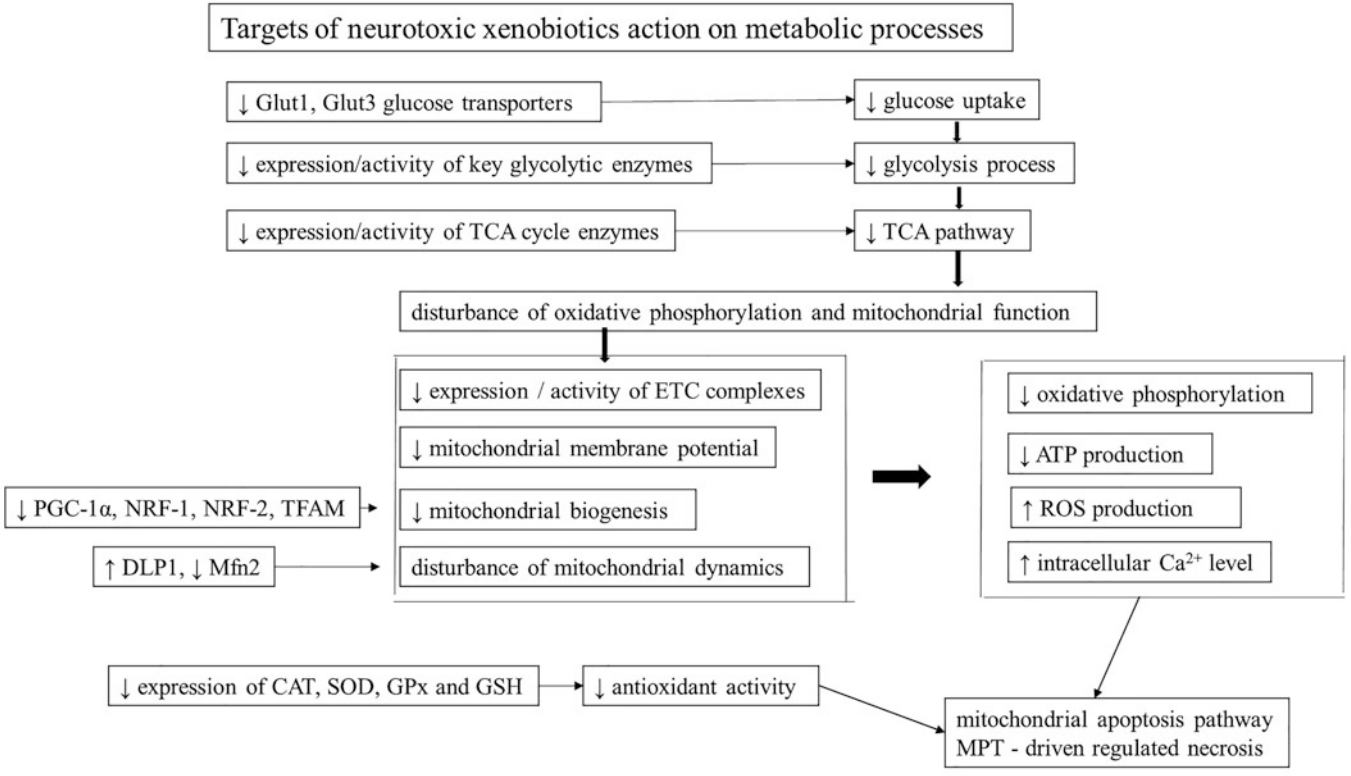

Fig. 6 A simplified diagram presenting the sites of action of neurotoxic xenobiotics on metabolic processes. Targets of neurotoxic xenobiotic action of on metabolic processes. CAT, catalase: DLP1, dynamin-like protein 1; ETC, electron transport chain; GPx, glutathione peroxidase; GSH, glutathione; Mfn2, mitofusin 2; MPT, mitochondrial permeability transition; NRF-1, nuclear respiratory factor 1; NRF-2, nuclear factor erythroid 2-related factor 2; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; ROS, reactive oxygen species; SOD, superoxide dismutase; TCA cycle, tricarboxylic acid cycle; TFAM, mitochondrial transcription factor A

potential; lowers the activity of complex II in ETC; and inhibits ATP production (Chen et al. 2019). High exposure to manganese causes mental and motor disorders similar to those observed in PD; however, due to differences in clinical symptoms, L-DOPA sensitivity, and damage to dopamine neurons in other regions of the brain, the link between manganese intoxication and PD is still controversial. The biomarker of manganese damage to dopamine neurons may be an increase in the level of prolactin in the blood, because dopamine inhibits the secretion of this hormone.

Metabolic Disturbances in the Neurotoxic Effects of Pesticides

A large body of data indicates that inhibition of complex I in ETC is a significant disturbance in the pathogenesis of PD. Pesticides such as rotenone and pyridaben are strong inhibitors of the activity of complex I and because of this action reduce oxygen consumption and cause structural damage to the mitochondria. They are highly lipophilic compounds and easily cross the BBB. Epidemiological studies have shown that exposure to rotenone increases the risk of developing PD, and this compound is often used in preclinical studies in animal models of this disease. The main mechanisms of the neurotoxic damage to dopaminergic neurons by rotenone include disruption of the mitochondrial ETC, inhibition of oxidative phosphorylation, enhancement of oxidative damage, excitotoxicity associated with disruption of calcium homeostasis, and enhancing the aggregation of α-synuclein (Richardson et al. 2019). In the case of paraquat, a frequently used herbicide that increases the risk of developing PD approximately twice, its influence on the dynamics of mitochondria has been demonstrated. This compound enhances expression of dynamin-like protein 1(DLP1), lowers mitofusin 2 (Mfn2) expression that intensifies the fission process of mitochondria, and leads to their fragmentation and loss of dopaminergic neurons in the substantia nigra pars compacta (Zhao et al. 2017). Moreover, overexpression of Mfn2 inhibits paraquat-induced OS and degeneration of dopamine neurons.

Sevoflurane: Disturbances of Glucose Metabolism and Iron Homeostasis

Postoperative cognitive dysfunction is often observed, especially in the elderly people, after the use of anesthetics. Studies on the mechanism of the neurotoxic effect of the inhalation anesthetic – sevoflurane – showed that this compound disturbs the metabolism of both iron and glucose. Sevoflurane has been shown to inhibit glucose uptake by cerebral microvascular endothelial cells, reduces the expression of some glycolysis and citric acid cycle enzymes, lowers mitochondrial respiration capacity, reduces ATP content in brain cortex and hippocampus, and causes the iron overload (Ge et al. 2021).

There are no specific biochemical markers indicating energy disturbances only within the CNS; however, studies based on PET and single-photon emission

computed tomography (SPECT) may provide evidence of impairment of brain glucose metabolism.

Excitotoxicity Induced by Xenobiotics

Glutamate (Glu) released from excitatory neurons evokes an action potential in the postsynaptic neurons and thus orchestrating fast glutaminergic neurotransmission and also long-lasting changes in the neural network, which are necessary for memory formation, learning, or cognition. Glu released into synaptic cleft binds to postsynaptic Glu receptors, ionotropic (NMDA, kainite, AMPA), metabotropic G-protein-coupled receptors and evokes intracellular signaling pathway. Glu can be toxic if the concentrations get too high; thus, its level is precisely controlled by excitatory amino acid transporters (EAAT1–5) expressed at highest levels on astrocytes (EAAT1–2) and on neurons (EAAT3). As Glu toxicity is due to excessive excitation of neurons expressing Glu receptors, it has been called "excitotoxicity." This mechanism is known to be important in diseases such as traumatic brain injury, brain ischemia, epilepsy, and neurodegenerative diseases (Gut et al. 2013). Excitotoxicity matters as overstimulation of weakened cells may cause them to die, thereby increasing the volume of the lesion or progression of specific disease. Overstimulation of NMDAR ameliorates reactive oxygen and nitrogen species production, acute mitochondrial dysfunction, and cellular energetic imbalance. Loss of ATP production results in the inhibition of Glu reuptake mechanisms that further augments Glu overload in the synaptic cleft. Based on this, it can be seen as surprising that NMDAR antagonists failed to be beneficial in clinical trials related to diseases with enhanced Glu neurotransmission. This suggests that the involvement of Glu in neuron damage is more complicated than the one presented by excitotoxicity concept. The mechanism of excitotoxicity is presented in Fig. 7. The current hypothesis is that the target of the future therapy of diseases with excitotoxicity component is not to block Glu transmission, but rather to restore the intensity of this neurotransmission to physiological levels.

There are several xenobiotics and environmental factors affecting Glu neurotransmission and/or inducing excitotoxicity. The mechanism in which xenobiotics evoke excitotoxicity is ambiguous. Tributyltin (TBT) is a compound used as a heat stabilizer and pesticide. It has been observed that mammal's exposure to TBT resulted in amnesia, epilepsy, memory impairments, and increase of intracellular calcium. Then, it has been proven that TBT enhances neuronal death through Glu receptor-dependent pathway and ERK-MAPK phosphorylation but also via p38-MAPK activation. Studies revealed that TBT in a concentration-dependent manner stimulated Glu release in cultured rat cortical neurons that correlated with cell viability, whereas inhibition of Glu receptors with MK801 (dizocilpine) or CNQX (cyanquixaline) prevented such effect. Moreover, inhibition of voltage-dependent calcium channels (VDCC) with nifedipine has also protected neurons from Glu toxicity. Since VDCC mediates Ca^{2+} currents after NMDAR stimulation,

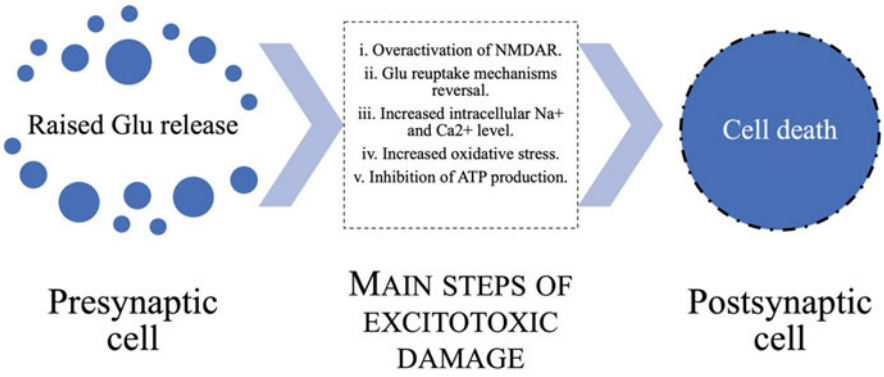

Fig. 7 The mechanism of excitotoxicity. Excess glutamate in the synaptic cleft evokes a cascade of processes in the postsynaptic cell leading to the cellular death. Glu – glutamate; NMDAR – N-methyl-D-aspartate receptor

studies of Nakatsu et al. confirmed the pro-excitotoxic effect of TBT (Nakatsu et al. 2006).

Herbicides such as atrazine (AT) and fungicides such as mancozeb (MZ) are known endocrine-disrupting compounds, whose noxious effect toward CNS has been long postulated. AT shows its neurotoxic potential on dopaminergic neurons of basal ganglia, which disturbs dopamine turnover (Li et al. 2014). MZ affects primarily dopaminergic and GABAergic neurons, which disturb mitochondrial functions and uncouple electron transport in respiratory chain (Domico et al. 2007; Negga et al. 2012). Current studies using cortical neuronal culture showed that both compounds massively increased the excitation/inhibition ratio (the ratio of levels of aspartate+Glu/glycine+GABA). This effect correlated with morphological changes of neurons, mitochondria damage, increased ROS level, vacuolization, and autophagy indicating neuronal damage, which might be associated with pro-excitotoxic effect of AT and MZ (Peña-Contreras et al. 2016).

Excitotoxicity is a main destructive effect of chronic alcohol usage. The precise mechanistic pathway of the neurodegeneration development during chronic alcohol consumption is still under debate; however, data suggest that overstimulation of NMDAR and decreased activation of GABAA receptor are key factors. Acetate, a product of ethanol metabolism, increased cell death, ROS production, TNFα, and cytosolic calcium level in PC12 cell line. Application of NMDAR blocker, memantine, decreased calcium concentration and TNFα that suggest that excitotoxicity is an important component of neurotoxic effect of ethanol (Chapp et al. 2019).

3,4-Methylenedioxymethamphetamine (MDMA) is an amphetamine derivative known for its degenerative effects to serotonergic and GABAergic neurons. Currently, limited data show that MDMA causes a significant and delayed increase in the concentration of Glu in the hippocampus. Both in vitro and animal model–based studies show that blockade of NMDAR reveals neuroprotective activity against

neurotoxicity induced by MDMA. However, detailed and extensive research on the excitotoxic mechanism of MDMA-induced neurotoxicity is still missing (Moratalla et al. 2017).

Glyphosate-based herbicides (GBH) are compounds with a proven potential to induce neurodegenerative effect in the mechanism of excitotoxicity. Current study showed that animals exposed even to a single oral dose of GBH demonstrated altered brain amino acid metabolism. In this study, GBH caused elevation of Glu, aspartate, and ornithine without degenerative effect on BBB, which suggests typical central effect of this compound. This report is in agreement with other studies demonstrating that subchronic exposure to GBH resulted in increased Glu release into synapse, reduced Glu uptake, and stimulated calcium influx in the hippocampus that may suggest persistent excitotoxicity process. Reduced Glu uptake correlated with increased serum concentration of S100 calcium-binding protein β (S100β), which is a marker of astrocyte degeneration, cells that are mainly responsible for Glu clearance. What is more, GBH may directly interact with NMDAR, leading to calcium flow into hippocampal neurons and boosting the cascade of processes of excitotoxicity (Cattani et al. 2017; Limberger et al. 2020).

Air pollution, including fine particulate matter (PM 2.5), is often reported as a factor closely associated with neuroinflammation and synapse dysfunction. Indeed, it has been observed that exposure of mouse pups to PM < 2.5 evoked excitotoxicity, neuroinflammation, and OS that persisted until adulthood with sustained increase in Glu level in the hippocampus. The noxious, excitotoxicity-related effect of PM 2.5 was also confirmed in human-derived primary neurons. Neurons exposed to PM 2.5 showed significantly reduced viability. In this experiment, addition of conditioned medium from microglia exposed to PM 2.5 further reduced viability; however, the neurotoxic process was ceased by pretreatment with MK801 that suggest that air pollution activates microglia, which is producing mediators evoking excitotoxicity (Li et al. 2018; Morris et al. 2021).

Exposure to the environmental factors and xenobiotics is a lifelong process. On the other hand, first clinical symptoms of, i.e., neurodegenerative disease in which excitotoxicity plays crucial role appear when molecular mechanisms of diseases are advanced. As such, it is of great importance to determine specific and sensitive biomarkers of pathological processes including excitotoxicity. Unfortunately, up to now, there are no ideal peripheral biomarkers of excitotoxicity with appropriate specificity and sensitivity. S100β is expressed mainly by astrocytes and is considered as an early marker of excitotoxic damage. The concentration of S100β is related with the severity of excitotoxicity; however, the detailed mechanism of S100β release in response to Glu overload is still unknown (Mazzone and Nistri 2014).

Types of Xenobiotic-Induced Nerve Cell Death

Our perception of the concept of cell death and its variants has changed over time. The frequent classification of the types of cell death was based on several aspects: 1) whether it is favorable/random/regulated/programmed; 2) whether it is accompanied

by phagocytosis, inflammation, or rupture of the plasma membrane; 3) whether it affects other neighboring cells; 4) whether it is an initiating stimulus death that comes from outside or inside the cell; 5) what morphology the cell has; and 6) what is the mechanism and biochemical pathway of death. Since 2005, the Nomenclature Committee on Cell Death has been trying to collect and refresh data on the distinction of cell death and according to the last update from 2018 recommends moving away from the morphological classification of death and dividing it into regulated cell death (RCD) and accidental cell death (ACD). This randomness in ACD is understood as the occurrence of extreme physicochemical or mechanical stimuli that lead to immediate, uncontrolled death. In contrast, regulation in RCD results from a genome-encoded mechanism, which can therefore be manipulated, including pharmacological or genetic interventions. RCD may be part of the physiological adaptation of the cell (as programmed cell death) or result from environmental disturbances that make it difficult to restore and maintain cell homeostasis. RCD includes intrinsic apoptosis, extrinsic apoptosis, necroptosis, ferroptosis, pyroptosis, parthanatos, entotic cell death, NETotic cell death, lysosome-dependent cell death, MPT-driven necrosis, and immunogenic cell death. Interestingly, all the abovementioned RCDs can take on a whole range of morphological features – indicating an apoptotic, necrotic, pro-inflammatory and anti-inflammatory, or immunomodulatory mechanism (Galluzzi et al. 2018). Neurons are a unique type of cells that, after prenatal neurogenesis, must last for many years to be able to function properly. This fact makes the nervous system an easy target for all kinds of negative environmental stimuli, for example, in the form of xenobiotics. RCD in neurons, despite the fact that under physiological conditions, it participates in the processes of neuroplasticity of the brain by removing excess neurons, in its pathological form is one of the main causes of neurodegenerative diseases. There is ample evidence that xenobiotics may indirectly cause neurodegeneration in the brain by inducing cell death mechanisms. This section presents examples of selected types of nerve cell death induced by xenobiotics (Fig. 8).

Apoptosis is the best-known type of cell death, which can be induced by the external or internal pathway. The essential phase of intrinsic apoptosis is the permeabilization of the outer mitochondrial membrane (MOMP), which is controlled by pro- and anti-apoptotic proteins from the B-cell lymphoma-2 (Bcl-2) family (Moldoveanu et al. 2014). Anti-apoptotic proteins include, for example, Bcl-2, Bcl-XL, or Bcl-w, whereas pro-apoptotic proteins include BH3-interacting domain death agonist (Bid), Bcl-2-associated agonist of cell death (Bad), Bcl-2-like protein 11 (Bim), phorbol-12-myristate-13-acetate-induced protein 1 (NoxA), p53 upregulated modulator of apoptosis (PUMA), Bcl-2-associated X protein (Bax), and Bcl-2 antagonist/killer 1 (Bak). Simply put, activation of the inner pathway of apoptosis involves altering the expression and activity of the Bcl-2 protein, inducing an increase in the expression of pro-apoptotic Bcl-2 family proteins that interact directly with the mitochondrial membrane causing MOMP. After membrane permeabilization, cytochrome c outflows from the mitochondria into the cytoplasm and binds to the apoptotic protease-activating factor-1 (Apaf-1). Apaf-1 recruits procaspase-9 turning it into an active form, which in turn cleaves and activates the

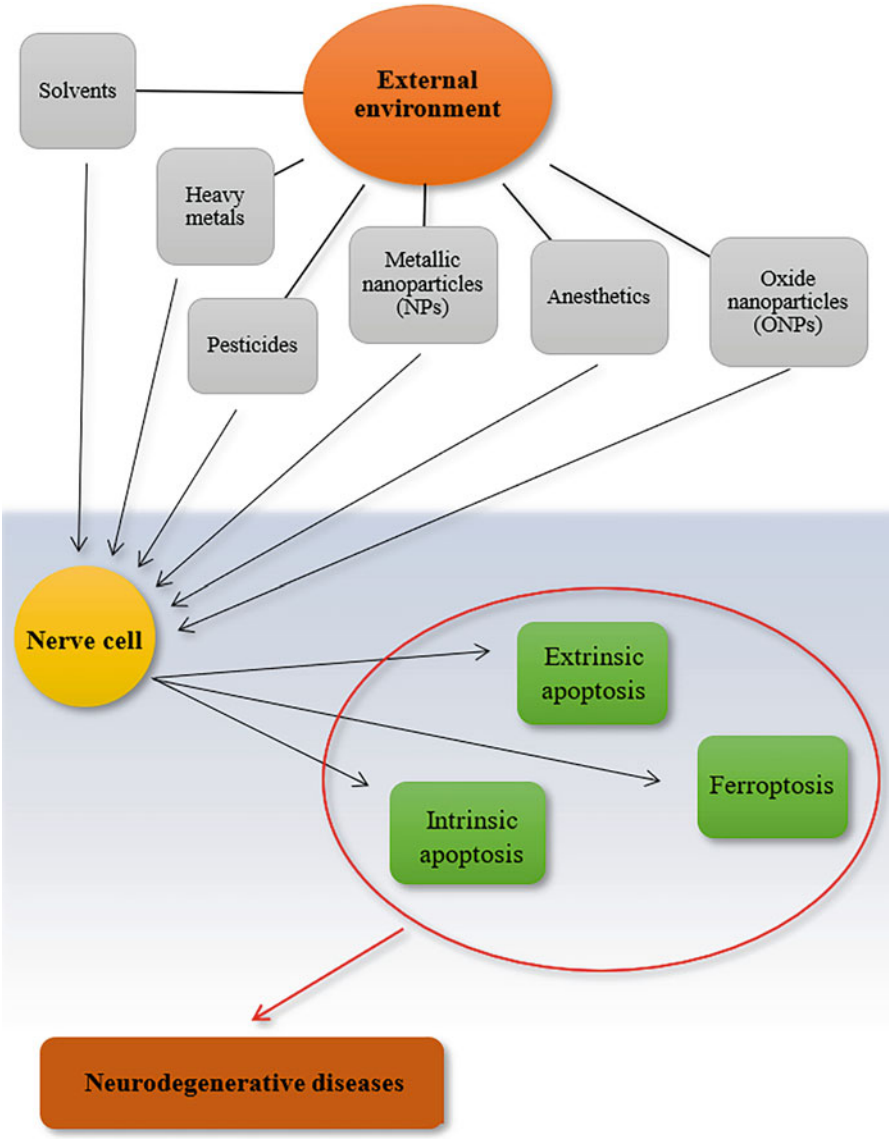

Fig. 8 Examples of xenobiotic-induced RCDs in nerve cell

effector caspases – caspase-3 and caspase-7 (Ribe et al. 2010). The external pathway of apoptosis is by ligating death receptors from the TNF family to the cell surface, resulting in activation of the Fas-associated death domain (FADD) protein. FADD captures cytoplasmic procaspase-8 molecules, turning them into their active form, which in turn can recruit other effector caspases or indirectly interact with the Bid

proteins. The Bid proteins, undergoing a series of changes, eventually activate the Bax proteins that cause MOMP (Micheau and Tschopp 2003). The literature provides some evidence of how xenobiotics can activate the processes of apoptosis in neuronal cells. Paraquat is one of the commonly used herbicides. It has been proven that this herbicide can activate the apoptosis of neuronal cells, mainly through its internal pathway. Paraquat triggers outflow of cytochrome c from the mitochondria and activates caspase-9 involving the pro-apoptotic proteins Bax and Bak (Fei et al. 2008). Another compound from the group of herbicides, glyphosate, also induces apoptotic processes in neuronal cells. Gui et al. (2012) showed that this compound induces apoptosis of PC12 differentiating neuronal cells by increasing the expression of Bax proteins. Metallic nanoparticles (NPs) are widely used in the food, cosmetic, and construction industries. Increasingly, new evidence shows that the widespread use of NPs can accumulate in the body and pose a huge risk to individual organs, including the brain. Song et al. (2016) included a number of evidence in their review of the risks associated with NP exposure. It turns out that TiO_2 NPs can induce apoptosis in the mouse hippocampus by increasing the expression of proteins crucial for this type of cell death, i.e., caspase-3, caspase-9, Bax, and cytochrome c, while reducing the expression of anti-apoptotic Bcl-2, indicating thus the intrinsic mechanism of apoptosis induction. Similar observations were made of the accumulation of TiO_2 NPs in the brain of rats, where the analysis also indicated an increase in the level of pro-apoptotic proteins, involving mitochondrial-dependent apoptosis. Interestingly, exposure to TiO_2 NPs during pregnancy led to an increase in the expression level of genes directly involved in apoptotic processes in the offspring, thus confirming that these particles exhibit neurotoxicity both during prenatal development and in adulthood. In the same review, information about other NPs that exhibit neurotoxic properties can be found. For example, silver NPs caused an increase in the number of apoptotic cells in the rat hippocampus and induction of apoptosis in human embryonic neural precursor cells. Gold and aluminum NPs increased the level of caspase-3 expression in the rat hippocampus and mouse brain, respectively.

Ferroptosis is newly described form of iron-depended RCD, which is morphologically and biochemically different from apoptosis, necrosis, necroptosis, and autophagic cell death. Ferroptosis induction is caused by excessive peroxidation of membrane lipids, promoted by reduced iron (Fe2) and inhibited by GPx 4. GPx 4 depends on level of GSH, which in turn depends on the activity of cystine/glutamate antiporter (xc^-). Moreover, inhibition of GPx 4 and xc^- can trigger ferroptosis (Fricker et al. 2018). Recent data showed that the isoflurane, one of the commonly used anesthetic, can induce ferroptosis in mouse hippocampus by downregulation of the xc^- system (Liu et al. 2021). On the other hand, exposure of primary neuronal cells to isoflurane may trigger ferroptosis by inhibiting the expression of GPx 4 (Xia et al. 2019). Zinc oxide nanoparticles (ZnONPs) are widely used nanomaterials in industry. Humans can be exposed to these molecules through inhalation, and because of their extreme small size, they can reach the brain causing damage in neuronal cells. One of the studies demonstrated that pulmonary exposure to ZnONPs induced ferroptosis in the neuronal cells of mouse cerebral cortex as well as in cultured neuron-like PC-12 cell line. Interestingly, the same study showed that using of ferrostatin-1, the specific ferroptosis inhibitor, could restrain neuronal cell death induced by ZnONPs both

in vivo and in vitro (Qin et al. 2020). A recent study has shown that cobalt (Co) NPs and Co salts can also affect neural cell death similar to ferroptosis. Exposure of human neuroblastoma cell line SH-SY5Y to Co NPs and $CoCl_2$ activates dose-dependent ferroptosis via lipid peroxidation and downregulation of GPx 4 expression in mRNA as well as in protein level (Gupta et al. 2020).

Conclusion

Much data shows that environmental pollution is an important factor causing damage to the function or morphology of nerve cells. Currently, many compounds with neurotoxic activity have been identified, and their mechanisms of action were investigated, but humans are exposed to many of these compounds simultaneously, and the combined effects of these compounds are very poorly understood, but limited data indicate that they may exert a synergistic effect. In addition, exposure to neurotoxic compounds in the prenatal and early postnatal periods can be particularly dangerous, because at this time the processes of progenitor cell proliferation, neuronal differentiation, and migration, as well as synaptogenesis and myelination, are most intense. Since changes in brain cells appear many years before clinical manifestations of neurodegenerative diseases, it would be desirable to assess early changes. For many neurotoxic xenobiotics, it is possible to assess the exposure to these compounds by measuring the levels of the parent compound or its metabolites in blood and/or urine. However, the assessment of early disturbances in the function or morphology of brain cells is not yet specific enough. Therefore, it should be emphasized that majority of the biomarkers of cell damage determined in biological fluids, presented in this chapter, are not generally specific only for brain cells. Performing PET and SPECT studies in humans in order to identify metabolic disturbances and neuroinflammation markers are so far rarely used for diagnostic purposes; rather, these methods are still being developed in order to select the appropriate radiotracers.

In addition to the difficulties in early diagnosis of nerve cell damage, another huge problem is the treatment of neurodegenerative diseases, because there are currently no effective, causative neuroprotective drugs. Thus, intensive research is needed to develop effective drugs that can inhibit the main mechanisms leading to damage to nerve cells. One promising line of research is an attempt to reduce the transfer of xenobiotics into the brain, by increasing the expression and activity of P-glycoprotein (P-gp) in the endothelial cells at the BBB. P-gp is an efflux transporter belonging to ATP-binding cassette transporter family, located on blood-tissue barriers. P-gp located at the BBB removes many xenobiotics from the brain into the circulation, and its inductors are examined for application in the therapy of neurodegenerative diseases (García-Varela et al. 2021). Moreover, the determination of P-gp expression by PET may be a marker of the resistance/susceptibility of a tested person to exposure to neurotoxic xenobiotics. There is also promising research into new multifunctional compounds that may prove to be more effective than those currently in use, as they should weaken/inhibit two or more mechanisms that lead to neuronal damage (Wichur et al. 2021).

Mini-dictionary of Terms

Excitotoxicity – This is the phenomenon of excessive glutamate neurotransmission; raised presynaptic glutamate release results in the synaptic cleft overload and NMDAR-mediated hyperactivation of postsynaptic neurons that initiates cascade of processes leading to cellular degeneration.

Free radicals – These are unstable and highly reactive molecules that contain unpaired electron; they are capable to react with macromolecules in cells (lipids, proteins, carbohydrates, DNA) and consequently lead to cell damage and death.

Inorganic arsenic – Chronic exposure to arsenic leads to a weakening of oxidative phosphorylation, the process of mitochondrial biogenesis, and a decrease in the potential of the mitochondrial membrane, increases the production of β-amyloid and β-secretase activity, and induces the process of apoptosis. Inorganic arsenic has been postulated to contribute to the pathogenesis of AD.

Neuroinflammation – This is a complex inflammatory response to various damaging triggers, e.g., trauma, autoimmunity, stroke, or toxin, mediated by cytokines, ROS, and other inflammatory factors, which are produced by all kinds of cells residing in the CNS but predominantly microglia.

Oxidative stress – This is a pathophysiological state, which is characterize as a disturbance in the balance between production of FRs (mainly ROS and RNS) and antioxidant defenses in biological systems.

Paraquat – This is a frequently used herbicide that increases the risk of PD by damaging dopamine neurons mainly by disrupting the dynamics of the mitochondria; this compound intensifies the process of mitochondrial fission, leading to mitochondria fragmentation.

Rotenone – This is a pesticide that inhibits complex I in ETC, and exposure to this compound increases the risk of PD; the main mechanisms of the neurotoxic damage to dopaminergic neurons by rotenone include disruption of the mitochondrial ETC, inhibition of oxidative phosphorylation, enhancement of oxidative damage, excitotoxicity associated with disruption of calcium homeostasis, and enhancing the aggregation of α-synuclein (Richardson et al. 2019).

Key Facts About Mechanisms of Neurotoxicity and Their Biomarkers

1. OS results in lipid peroxidation, damage to proteins, nucleic acids, carbohydrates, disruption of Ca^{2+} homeostasis, reduction of ATP levels, and, consequently, dysfunction and subsequent cell death.
2. Due to the high consumption of oxygen and high content of lipids, CNS is particularly vulnerable to damage caused by OS.
3. Chronic or exaggerated neuroinflammatory response results in dysregulated overproduction of proinflammatory cytokines and involves adaptive immunity.
4. The pivotal moment in neuroinflammation is microglial activation.

5. Neurotoxic xenobiotics most often disrupt oxidative phosphorylation by inhibiting the expression or activity of electron transport chain complexes.
6. Some neurotoxic xenobiotics can disrupt oxidative phosphorylation by influencing the potential of the mitochondrial membrane, the process of mitochondrial biogenesis, and the dynamics of mitochondria (fusion-fission processes).
7. Xenobiotics-induced excitotoxicity may initiate neurodegenerative changes in the CNS.
8. The influence of specific xenobiotics on RCDs is associated with the initiation of the death mechanism in various ways, depending on environmental conditions and the form of exposure.

Summary Points

1. The main mechanisms of neurotoxicity of xenobiotics include induce OS, neuroinflammation, metabolic disturbances, and excitotoxicity of Glu.
2. OS may play an important role in the development of neurodegenerative diseases, as well as psychiatric disorders.
3. Many xenobiotics can disrupt metabolic processes, but most of the evidence concerns the involvement of this mechanism in the neurotoxic effects of heavy metals and pesticides.
4. Products of cellular component oxidation are important biomarkers of OS caused by xenobiotics.
5. Among numerous biomarkers of OS, only a few are considered specific to CNS-related damage.
6. Markers of microglia activation, as well as cytokine profiling, are frequently used in animal studies of xenobiotic action.
7. PET is a widely used technique based on a radiolabeled molecule characterized by the ability to cross the BBB as well as high affinity and selectivity in tandem with low nonspecific binding.
8. To date, a lot of neuroinflammation markers – molecular targets for radiotracers – have been described, but the most popular is TSPO.
9. Until now, there are no specific and sensitive enough peripheral biomarkers of excitotoxicity.
10. It is difficult to unambiguously assign one specific type of cell death responsible for the occurrence of the neurodegenerative diseases.
11. There is a whole network of connections between the mechanisms of various types of neuronal cell death, which to this day are not fully understood.

Cross-References

► Autophagy as a Biomarker of Cytotoxicity
► Biomarkers of $PM_{2.5}$ Exposure: Use of Metabolomics as a Platform
► Biomarkers of Lead Exposure: Platforms and Analysis

- ▶ Blood-Brain Barrier Function as a Biomarker in Toxicology: Impact of Environmental Toxicants
- ▶ DNA Adducts as Biomarkers in Toxicology
- ▶ DNA Methylation as a Biomarker and Application to Aluminum: *ADRB2* 5′-Untranslated Region (5′-UTR) Methylation Level
- ▶ Oxidative Stress Biomarkers and Their Applications to Detect Excessive Fluorine
- ▶ Pro-inflammatory Markers of Environmental Toxicants

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Part III

Genetic, Cellular, and Histological Variables

Genetic and Epigenetic Biomarkers Related to 2-Oxoglutarate/Fe(II)-Dependent Oxygenases and Implications for Disease and Toxicology

16

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Abstract

Biomarkers are important in investigating relationships among human biology, disease, environmental exposure, and toxicology. Genetic and epigenetic biomarkers, particularly the alkylated DNA and RNA, widely exist in cells and tissues and have attracted great attention on biomarker formation and regulation processes and their potential applications in the early detection of diseases. The present chapter primarily focuses on the biochemical and genetic properties of alkylated DNA/RNA biomarkers specifically related to the 2-OG/Fe(II)-dependent oxygenases, including AlkB family dioxygenases (such as *E. coli* AlkB and its mammalian homologs ALKBH1–8 and FTO) and mammalian TET (TET1–3)/JBP (JBP1 and 2) family proteins, and the implications of these markers to disease and toxicology. In addition, spectroscopic and computational studies have been carried out in line with biochemical studies on the 2-OG/Fe(II)-dependent oxygenases and nucleic acid biomarkers, providing further insights into their mechanisms of action.

Keywords

Genetics · Epigenetics · DNA/RNA biomarker · 2-OG/Fe(II)-dependent oxygenase · AlkB family · ALKBH1–8 · FTO · TET/JBP family · Spectroscopic · Computational · Toxicology

Abbreviations

2-OG	2-Oxoglutarate
DNMT	DNA methyltransferase
ds-DNA	Double-strand DNA
FTO	Fat mass and obesity-associated protein
MGMT	O^6-Methylguanine-DNA methyltransferase
MMS	Methyl methanesulfonate
SAM	*S*-Adenosylmethionine
ss-DNA	Single-strand DNA
TET	Ten-eleven translocation
WTAP	Wilms' tumor 1-associating protein

Introduction

A biomarker is an objective measure that indicates a normal or abnormal process taking place in the body at a given moment. Biomarkers play important roles in elucidating relationships among environmental exposure, human biology, disease, and toxicology. Numerous molecules have been reported on and used as biomarkers, such as DNAs, RNAs, proteins, and hormones (Dhama et al. 2019).

DNA/RNA alkylation, particularly methylation, is a biological process that results in covalent modification of the nucleobases without changing the sequence. This process usually results from endogenous and exogenous agents (Friedberg et al. 2004). On one side, the unwanted alkylated bases or adducts could be toxic and mutagenic (Friedberg et al. 2004; Fedeles et al. 2015). On the other side, as a form of epigenetic modification in cells, methylation has an important effect on the maintenance of genome structure and regulation of gene expression (Petryk et al. 2021). Methylation influences the transcription of many tumor suppressor genes critical to cancer initiation and progression (Petryk et al. 2021). Thus, organisms have evolved multiple pathways to remove or otherwise regulate alkylated DNA/RNA (Schermerhorn and Delaney 2014; Jeggo et al. 2016; Chatterjee and Walker 2017). Direct reversal repair, including many enzymes from the 2-oxoglutarate (2-OG)/Fe(II)-dependent oxygenases family, is a straightforward and energy-efficient repair mechanism, whereby unwanted alkylated DNA/RNA biomarkers are repaired directly by a group of direct reversal repair enzymes (Sedgwick 2004; Mishina et al. 2006; Sedgwick et al. 2007; Zheng et al. 2014; Fedeles et al. 2015; Chatterjee and Walker 2017). Besides repair, there are additionally innate modifications such as 5-methylcytosine (m5C) and N^6-methyladenine (m6A) that are regarded as epigenetic markers that also impact health and disease (Wu and Zhang 2010; Meyer and Jaffrey 2014; Hashimoto et al. 2015; Luo et al. 2015; Zhao et al. 2017; Zaccara et al. 2019; Wardowska 2021).

2-Oxoglutarate/Fe(II)-dependent oxygenases are a major class of non-heme iron proteins that have been recognized as a widely distributed superfamily in aerobic biology (Pastor et al. 2013; Hausinger and Schofield 2015; Lu et al. 2015; Markolovic et al. 2015; Islam et al. 2018). 2-OG/Fe(II)-dependent oxygenases employ Fe(II) ion as a cofactor and 2-OG and O_2 as co-substrates with release of CO_2 and succinate during catalysis (Fig. 1). These enzymes catalyze a wide range of reactions including dealkylation, desaturation, and halogenation, and their catalytic activities are reflected in their biological functions (Martinez and Hausinger 2015). 2-OG/Fe(II)-dependent oxygenases participate in transcriptional regulation, secondary metabolite biosynthesis, fatty acid metabolism, and nucleic acid modification/repair (Islam et al. 2018). Moreover, these enzymes are also regarded as agrochemical targets in plants and are under consideration as therapeutic targets for cancer and anemia in humans (Islam et al. 2018).

The present chapter focuses primarily on alkylated DNA/RNA biomarkers specifically related to the 2-OG/Fe(II)-dependent AlkB family dioxygenases (including *E. coli* AlkB and its mammalian homologs ALKBH1–8 and FTO) and mammalian TET (TET1–3)/JBP (JBP1 and 2) family proteins and their implications to disease and toxicology. DNA biomarkers are discussed in section "DNA Biomarkers," RNA

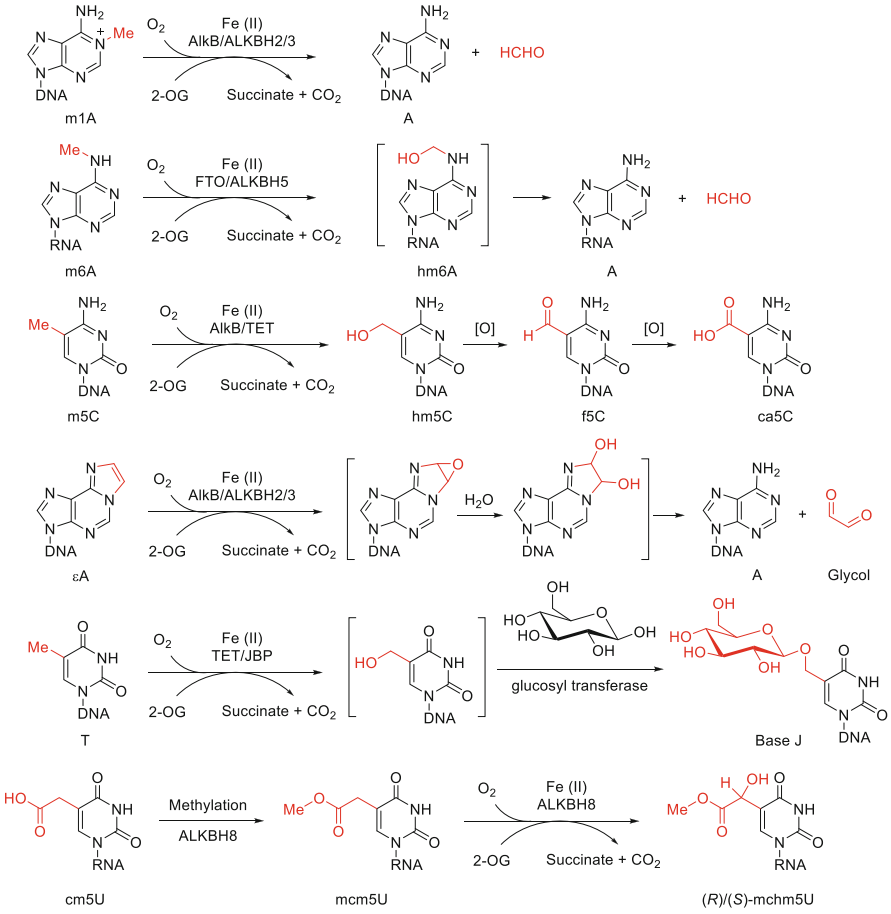

Fig. 1 Proposed mechanisms of oxidative modification reactions on representative DNA/RNA substrates catalyzed by 2-OG/Fe(II)-dependent oxygenases

biomarkers in section "RNA Biomarkers," 2-OG/Fe(II) proteins in section "AlkB Family and TET/JBP Family Enzymes," spectroscopic and computational studies in section "Brief Summary of Spectroscopic and Computational Studies," and implications of genetic/epigenetic biomarkers in human diseases and toxicology in section "Applications to Prognoses, Other Diseases, or Conditions."

DNA Biomarkers

DNA biomarkers are detectable, repeatedly generated, and relatively stable in tissues or fluids, and they can be identified and measured. They have the potential to be used as clinical indicators for the diagnosis and prognosis of various diseases, prediction of therapeutic response, monitoring tumor progression, and toxin exposure (Lu et al. 2017).

3-Methylcytosine (m3C) and 1-Methyladenosine (m1A)

m3C and m1A (Fig. 2) can be chemically generated via non-enzymatic methylation, either from endogenous SAM (Barrows and Magee 1982) or exogenous methylating agents, such as MMS, dimethylsulfate, and methyl iodide.[2] They are predominantly generated in ss-DNA during replication and transcription. In ds-DNA, the reactive site is involved in base pairing and thus likely protected from modification (Bodell and Singer 1979). m3C and m1A lesions have been shown to be efficiently repaired via direct oxidative reversal by AlkB, ALKBH2, and ALKBH3 (Fig. 1) (Falnes et al. 2002; Trewick et al. 2002; Aas et al. 2003). Kinetic studies reveal that AlkB and ALKBH3 prefer repairing ss-DNA, whereas ALKBH2 is more efficient in ds-DNA (Yang et al. 2008; Chen et al. 2017). More recently, ALKBH1 has been shown to be a functional mitochondrial AlkB homolog that repairs m3C in ss-DNA (Westbye et al. 2008). It is noteworthy that the minimal substrate is defined as 1-methyl-dAMP(5′), requiring a phosphate 5′ of the damaged base because the nucleosides of m1A and m3C only stimulate 2-OG turnover but are not demethylated (Koivisto et al. 2003; Welford et al. 2003).

1-Methylguanine (m1G) and 3-Methylthymine (m3T)

m1G and m3T (Fig. 2) are adducts in which the methyl group is on the Watson-Crick side of nucleobase rings. Such positioning unavoidably interferes with hydrogen-bond pairing. To the extent that the methyl group is left unrepaired, it creates opportunities for replication block and mutagenicity (Delaney and Essigmann 2004). AlkB and ALKBH2 repair m1G and m3T, preferring ds-DNA, whereas ALKBH3 repairs m3T in both ss-DNA and ds-DNA (Koivisto et al. 2004; Chen et al. 2016). Both human and mouse FTO preferentially repair m3T in ss-DNA, showing negligible activity against m3T in ds-DNA (Gerken et al. 2007).

4-Methylcytosine (m4C) and 2-Methylguanine (m2G)

In contrast to m1G and m3T, m4C and m2G (Fig. 2) in DNA avoid disruption of Watson-Crick base pairing by allowing their methyl group to swivel away from the H-bond interface (Delaney and Essigmann 2004). In vitro, AlkB demonstrates the biochemical capability to demethylate these two adducts in ss-DNA (Li et al. 2013a).

O^6-Methylguanosine (O6mG)

O^6-Methylguanosine (O6mG, Fig. 2) has been linked to a form of severe DNA damage that causes G-to-A mutations in cell (Eadie et al. 1984). The demethylation of O6mG is carried out by MGMT, which cannot be regenerated after the reaction (Fan et al. 2013). Recently, an engineered AlkB (D135R, D135G, and D135A) as an

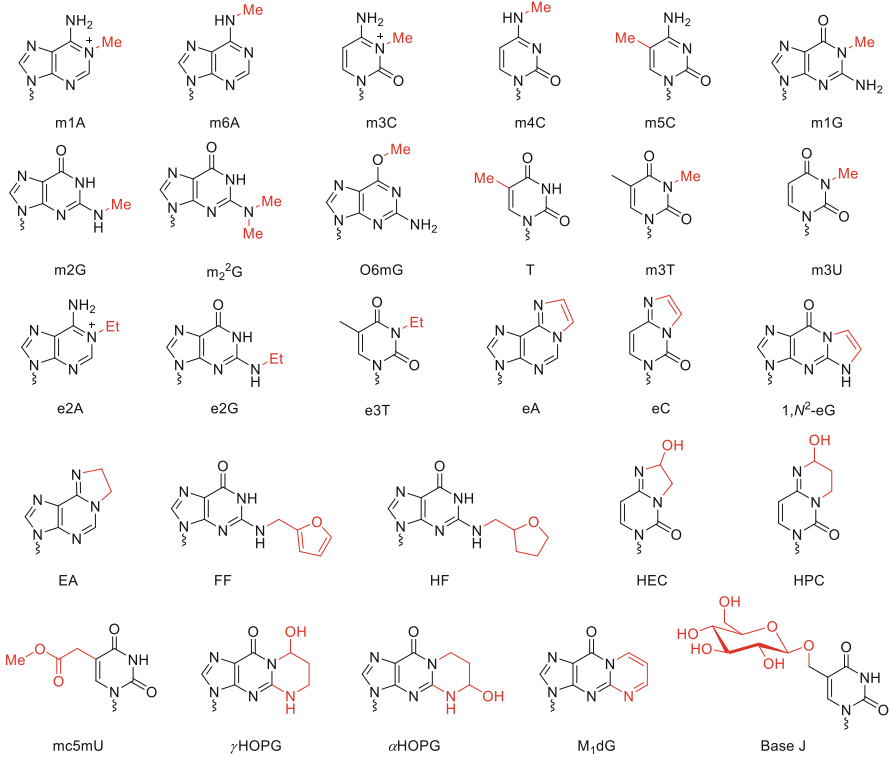

Fig. 2 The chemical structures of DNA/RNA biomarkers related to 2-OG/Fe(II) enzymes

exogenous source to demethylate O6mG in DNA was discovered by using a high-throughput screening method (Wang et al. 2021).

5-Methylcytosine (m5C), 5-Hydroxymethylcytosine (hm5C), 5-Formylcytosine (f5C), and 5-Carboxylcytosine (ca5C)

5-Methylcytosine (m5C, Fig. 2) has been shown to play crucial roles in genomic imprinting, X-chromosome inactivation, transposable element repression, and transcription regulation (Wu and Zhang 2017). As the predominant DNA modification in eukaryotes, m5C is commonly regarded as the "fifth base" in mammalian genomic DNA, mainly located at 60–80% of CpG dinucleotides (Klose and Bird 2006). Methyl groups can be transferred to the C-5 position of cytosine by DNMTs in the presence of SAM (Okano et al. 1999; Goll and Bestor 2005). TET1–3 promotes m5C in DNA to undergo successive oxidation to 5-hydroxymethylcytosine (hm5C), 5-formylcytosine (f5C), and 5-carboxylcytosine (ca5C) in vivo and in vitro (Figs. 1 and 2) (Tahiliani et al. 2009, p. 1; He et al. 2011; Ito et al. 2011; Yu et al. 2012; Zhang et al. 2014; Crawford et al. 2016; Tamanaha et al. 2016). The further oxidized f5C and ca5C can be recognized and excised by thymine DNA glycosylase coupled with the base excision repair process to regenerate the unmethylated cytosine (He et al. 2011; Maiti and Drohat 2011; Zhang et al. 2012a). Prior work has demonstrated that overexpression of the catalytic domain of human TET1 leads to a notable increase in the levels of hm5C, f5C, and ca5C in genomic DNA of HEK293T cells (Liu et al. 2013). TET proteins are also known to oxidize m5C in ss-DNA, although with lower activity comparing to ds-DNA (Zhang et al. 2012b; Kizaki and Sugiyama 2014). Kinetic studies suggest that TET2 is more specific on oxidation in DNA for m5C ($k_{cat}/K_m = 4.42 \times 10^3\ M^{-1}\ s^{-1}$) than for hm5C ($k_{cat}/K_m = 0.70 \times 10^3\ M^{-1}\ s^{-1}$) or f5C ($k_{cat}/K_m = 0.35 \times 10^3\ M^{-1}\ s^{-1}$). Therefore, hm5C is relatively stable, and the generation of hm5C can be a potentially stable marker for TET protein regulatory functions (Hu et al. 2015). More recent work has shown that AlkB, ALKBH2, and ALKBH3 oxidize m5C to hm5C, f5C, and ca5C in vitro (Bian et al. 2019).

N^6-Methyladenine (m6A)

N^6-Methyladenine (m6A, Fig. 2) in DNA (m6A in RNA is discussed in section "N^6-Methyladenine") is an emerging epigenetic marker in the mammalian genome. This DNA modification occurs in cell and tissue, co-localizing with H3K9me3-marked heterochromatin domains and is markedly upregulated in glioblastoma (Greer et al. 2015; Zhang et al. 2015; Koziol et al. 2016; Xie et al. 2018). The ALKBH1 enzyme demethylates m6A in unpairing regions (e.g., SIDD, stress-induced DNA double helix destabilization regions) of mammalian genome (Zhang et al. 2020). Recombinant ALKBH1 can efficiently demethylate m6A from ss-DNA through the detectable intermediate N^6-hydroxymethyladenine (hm6A, Fig. 1) (Zhang et al. 2020). However, enzymatic activities towards m6A in dual- or

hemi-methylated ds-DNA are markedly reduced, suggesting that demethylation may be coupled with transcription and/or replication in vivo (Wu et al. 2016). Enzymatic profiling studies have revealed that ALKBH1 prefers bubbled or bulged DNAs as substrates, instead of ss-DNA or ds-DNA, supported by enzymatic kinetic analyses with bulged DNA ($k_{cat}/K_m = 0.71 \times 10^3\ M^{-1}\ s^{-1}$) compared to ss-DNA ($k_{cat}/K_m = 0.46 \times 10^3\ M^{-1}\ s^{-1}$) (Zhang et al. 2020). In addition, ALKBH4 efficiently demethylates DNA duplex oligonucleotide containing m6A base modification within a consensus motif (5′-AGAAGAGGA-3′). For ss-DNA substrate, no effect of ALKBH4 was detected, even after prolonged incubations (Kweon et al. 2019).

Thymine and β-D-Glucopyranosyloxymethyluracil (Base J)

Thymine is a canonical base in DNA. Interestingly, work by Carell and colleagues showed that TET1–3 proteins oxidize thymine to 5-hydroxymethyluracil (hm5U) in mouse embryonic stem cell DNA (Fig. 1). The oxidation dynamics of thymine modifications in vivo can be monitored by quantitative mass spectrometry, using isotope-labeled nucleosides as internal standards. Interestingly, TET proteins do not seem to further oxidize hm5U to 5-formyluracil (f5U), which is more akin to ROS-induced DNA damage (Pfaffeneder et al. 2014).

β-D-Glucopyranosyloxymethyluracil (base J, Fig. 1) is a hyper-modified DNA base found in eukaryotes. This DNA modification is found within members of kinetoplastid protozoans and is abundant in their telomeres, where base J substitutes around 1% of the total T in the genome and is primarily present in repetitive DNA sequences, such as telomeric repeats (Cliffe et al. 2012). Base J is synthesized via a two-step reaction from thymine (Yu et al. 2007; Borst and Sabatini 2008; Cliffe et al. 2009). Step one involves the oxidative hydroxylation of thymine to hm5U by JBP1 and JBP2 utilizing a 2-OG/Fe(II)-dependent mechanism (Fig. 1). The newly generated hydroxyl group is subsequently conjugated with glucose by a glucosyl transferase to form base J (Fig. 1) (Cliffe et al. 2010). Within the organisms *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania*, base J acts as a RNA polymerase II transcription terminator, which can regulate gene expression and can ultimately prove lethal to the cell (Cliffe et al. 2012).

Other Simple Alkylated DNA Biomarkers

The AlkB family proteins can not only oxidize methyl-substituted DNA bases but also dealkylate other alkylated adducts, such as 1-ethyladenonine (e1A), 2-ethylguanine (e2G), N^2-furan-2-yl-methylguanine (FF), and N^2-tetrahydrofuran-2-yl-methylguanine (HF) (Fig. 2) (Duncan et al. 2002; Li et al. 2013a). ALKBH2 and ALKBH3 are reported to repair N^3-ethylthymine (e3T, Fig. 2), but not O^2-ethylthymine, and O^4-ethylthymine as well as N^3-carboxymethylthymine and O^4-carboxymethylthymine (You et al. 2016).

Exocyclic DNA Biomarkers

Etheno-DNA lesions (Fig. 2) are a type of highly mutagenic and toxic biomarker; they are formed from products of lipid peroxidation (LPO) and the carcinogen vinyl chloride (Bartsch and Nair 2000, 2006). Etheno-DNA lesions are efficiently induced in chronically inflamed human tissues as well as in rodents (Nair et al. 1995). Several etheno-DNA biomarkers have been characterized: 1,N^6-ethenoadenine (*εA*), 3,N^4-ethenocytosine (*εC*), 1,N^2-ethenoguanine (*1,N^2-εG*), and N^2,3-ethenoguanine (*N^2,3-εG*). These exocyclic biomarkers have been found to be repaired through direct reversal repair and base excision repair (Mishina et al. 2006; Schermerhorn and Delaney 2014). *E. coli* AlkB *was initially found to dealkylate* εA (Fig. 1) (Delaney et al. 2005; Mishina et al. 2005). Subsequently, the human enzyme ALKBH2 was shown to repair *εA*, *εC, and 1,N^2-εG* in ds-DNA, with less efficient repair of εA and εC in ss-DNA; ALKBH3 only repairs εC in ss-DNA (Chang et al. 2015; Zdżalik et al. 2015). The non-enzymatic binding of alkyladenine glycosylase to εC specifically blocks ALKBH2-catalyzed repair of εC in vitro, but not for methylated substrates, supporting ALKBH2 as a repair enzyme for the mutagenic εC lesion (Fu and Samson 2012). Although *N^2,3-εG* is known to cause G-to-A transition mutations during DNA replication in *E. coli* (Matijasevic et al. 1992), *N^2,3-εG* is not repaired by the AlkB family enzymes (Morinello et al. 2002; Chang et al. 2015). 1,N^6-Ethanoadenine (EA, Fig. 2), a DNA adduct formed by the anticancer drug, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), can be converted to undamaged adenosine by AlkB (Li et al. 2012), a finding consistent with biological data demonstrating that AlkB almost completely reverses EA toxicity in vivo (Frick et al. 2007). Moreover, 3,N^4-α-hydroxyethanocytosine (HEC), 3,N^4-α-hydroxypropanocytosine (HPC), and three propano-exocyclic lesions, guanine *α*-hydroxypropanoguanine (*α*HOPG), *γ*-hydroxypropanoguanine (*γ*HOPG), and malondialdehydeguanine (M_1dG), are also processed by *E. coli* AlkB in vitro (Fig. 2) (Maciejewska et al. 2010, 2013; Singh et al. 2014).

RNA Biomarkers

Genome is transcribed in the form of RNA, including RNA coding for proteins (~2% of the human genome) and noncoding RNA (~20% of the human genome) (Santosh et al. 2015). Compared with DNA biomarkers, RNA biomarkers have the advantage of providing dynamic insights into cellular states and regulatory processes (Xi et al. 2017). In addition, RNA is more readily present than DNA within cells. Many RNA modifications are conserved across eukaryotes (Liu and He 2017).

3-Methylcytosine and 1-Methyladenine

3-Methylcytosine (Fig. 2) was first discovered in total RNA from *Saccharomyces cerevisiae* (Hall 1963). In addition, m1A (Fig. 2) is an important posttranscriptional

modification that was first isolated from RNA (Dunn 1961). Previously, AlkB and ALKBH3 (but not ALKBH2) were reported to modify m3C and m1A in RNA, suggesting that these enzymes may play roles in RNA repair (Aas et al. 2003). Recently, ALKBH1 was demonstrated to be capable of demethylating m3C in mRNA of mammalian cells in vitro (Westbye et al. 2008; Ma et al. 2019). ALKBH1 is another tRNA demethylase that mediates the demethylation of m1A in tRNAs (Liu et al. 2016). The frequency of m1A in two mitochondrial tRNAs is consistently increased in ALKBH1 knockout cells (Kawarada et al. 2017). The ALKBH1-catalyzed demethylation of the target tRNAs results in attenuated translation initiation and decreased usage of tRNAs in protein synthesis (Liu et al. 2016). ALKBH7 demethylates a 35-base RNA probe consisting of a single m1A flanked by the mt-Leu1 sequence in vitro, with slightly lower activity towards ds-RNA than ss-RNA (Zhang et al. 2021).

3-Methyluracil (m3U)

FTO, a protein associated with human obesity, was shown to catalyze demethylation of m3U (Fig. 2) in ss-RNA ($k_{cat}/K_m = 0.014 \times 10^3\ M^{-1}\ min^{-1}$) with slightly higher efficiency than m3T in ss-DNA ($k_{cat}/K_m = 0.007 \times 10^3\ M^{-1}\ min^{-1}$) (Gerken et al. 2007; Jia et al. 2008).

1-Methylguanine and N^2,N^2-Dimethylguanine (m_2^2G)

1-Methylguanine in tRNA, introduced by chemical methylation, can be efficiently repaired by AlkB (Falnes 2004). N^2,N^2-dimethylguanine (m_2^2G, Fig. 2) is the most prominent case found in the majority of eukaryotic tRNAs at base pair m_2^2G26:A44, which originates from G26 in mitochondrial (mt) tRNAs and is methylated by the methyltransferase TRMT1 (Pallan et al. 2008; Dewe et al. 2017). m_2^2G disrupts Watson-Crick base pairing, which can alter interactions both within and between nascent mt-RNAs, as well as RNA-protein interactions. Recent work has demonstrated that the AlkB mutant (D135S/L118V) efficiently removes one methyl group from the m_2^2G and converts it to N^2-methylguanine (m2G). Unlike m_2^2G, m2G does not block reverse transcriptase reactions under sequencing conditions, thus not affecting tRNA sequencing efficiency (Dai et al. 2017). Additionally, human ALKBH7 demethylates mt-Ile m_2^2G26 within ds-RNA in vitro (mt-Ile m_2^2G26 regulates nascent polycistronic mt-RNA processing and mitochondrial function) (Zhang et al. 2021).

5-Methylcytosine, 5-Hydroxymethylcytosine, 5-Formylcytosine, 5-Hydroxymethyl-2′-*O*-Methylcytosine, and 5-Formyl-2′-*O*-Methylcytosine (f5Cm)

TET1–3 enzymes possess the ability to catalyze the transformation of m5C (Fig. 1) to hm5C in RNA in vitro. The catalytic domains of all three TET enzymes

could promote the formation of hm5C in human cells (Fu et al. 2014). hm5C is present at appreciable levels (~1 per 5000 of 5-methylcytosine) in the RNA of mammalian cells and tissues (Fu et al. 2014). Interestingly, Tet-mediated oxidation of m5C in RNA is much less efficient than that in duplex DNA. In addition, TET1 prefers ss-DNA to ss-RNA in the same sequence context (Fu et al. 2014). Moreover, overexpression of the catalytic domain of TET2 can promote hm5C levels and reduce m5C in tRNAs in comparison with wild-type cells (Shen et al. 2021). 5-Formylcytidine was first discovered in mammalian mitochondrial tRNA at position 34 of mt-tRNA^{Met} from vertebrates and nematodes (Moriya et al. 1994; Watanabe et al. 1994). Biogenesis of f5C34 is initiated by SAM-dependent methylation catalyzed by NSUN3 (Nakano et al. 2016). ALKBH1 first hydroxylates m5C34 to form hm5C34 and then oxidizes hm5C34 to f5C34 in the anticodon stem loop of mt-tRNA^{Met} in vitro. ALKBH1 is also involved in the biogenesis of hm5Cm and f5Cm at position 34 of anticodon in cytoplasmic tRNA^{Leu}. In the case of ct-tRNA^{Leu} (CAA), NSUN2 introduces m5C34 to initiate the formation of hm5Cm34/f5Cm34. By contrast, NSUN3 forms m5C34 to initiate f5C34 formation in mt-tRNA^{Met} (Haag et al. 2016; Kawarada et al. 2017).

N^6-Methyladenine

N^6-methyladenosine (Fig. 2) is a prevalent internal modification in mRNA and noncoding RNA affecting various cellular pathways, which constitutes 1–2% of all adenosine in mRNA (Perry et al. 1975). m6A in RNA has been characterized molecularly, biochemically, and phenotypically, including elucidation of the protein with functions of writers (METTL3, METTL14, and WTAP), erasers (FTO and ALKBH5), and readers (YTH family, hnRNP family, and KH/RM/RBD family). FTO as an RNA demethylase has efficient oxidative demethylation activity targeting the m6A residues in RNA in vitro (Fig. 1) (Jia et al. 2011). During the demethylation process, FTO oxidizes m6A to generate N^6-hydroxymethyladenine (hm6A) as an intermediate and N^6-formyladenine (f6A) as a further oxidized product. These two intermediates have half-lives of ~3 h in aqueous solution under physiologically relevant conditions and are present in isolated messenger RNA from human cells as well as mouse tissues (Fu et al. 2013b), which adds potential complexity to this demethylation regulation (He 2010).

ALKBH5 is another mammalian demethylase that oxidatively reverses m6A in mRNA in vitro and in vivo (Zheng et al. 2013). However, the reaction pathway seems to be different compared to that of FTO because ALKBH5 directly converts m6A to adenine without any observable intermediate. Differences in the active site residues between these two proteins, as well as the weaker interaction between Arg251 and 2-OG, may explain the slight differences in the demethylation process (Chen et al. 2014). Endogenously methylated m6A in tRNA was proposed to be demethylated by ALKBH3, but not in rRNA or mRNA, significantly enhancing protein translation efficiency (Ueda et al. 2017).

5-Carboxymethyluridine (cm5U) and 5-Methoxycarbonylmethyluridine (mcm5U)

In ALKBH8 knockout mice and mammalian cells, the methyltransferase domain of ALKBH8 has been shown to methylate 5-carboxymethyluridine (cm5U) to 5-methoxycarbonylmethyluridine (mcm5U) in vivo (Fu et al. 2010a; Songe-Møller et al. 2010). In 2010, He and colleagues reported that mcm5U undergoes hydroxylation to generate mchm5U at the anticodon wobble base by the 2-OG/Fe(II)-dependent domain of ALKBH8 (Fig. 1) (Fu et al. 2010b). Interestingly, a diastereomeric pair of wobble nucleosides in mammalian tRNA, (*R*)- and (*S*)-mchm5U are the hydroxylated forms of mcm5U; only the (*S*)-diastereomer is generated by ALKBH8. The (*R*)- and (*S*)-mchm5U are present in mammalian $tRNA^{Arg}$(U*CG) and $tRNA^{Gly}$(U*CC) (U* is the modified uridine), respectively (van den Born et al. 2011). This tRNA modification modulates the decoding selectivity of the codons of tRNA, thereby affecting translation in a codon-specific manner.

AlkB Family and TET/JBP Family Enzymes

AlkB is one of the four enzymes involved in the adaptive response to alkylation damage in *E. coli*. AlkB was first found to show a crucial response to demethylation in 1997 (Pan et al. 2008). In 2002, AlkB protein was identified as a 2-OG/Fe(II)-dependent dioxygenase that repaired alkylated DNA by demethylation (Fig. 1) (Falnes et al. 2002; Trewick et al. 2002). The active site of AlkB contains a two histidine (His-131 and His-187) and one aspartate (Asp-133) triad, typical for 2-OG/Fe(II)-dependent enzymes (Solomon et al. 2000). Firstly, these amino acids bind to a nonheme mononuclear Fe(II) with 2-OG and O_2 in the active site, ultimately leading to the formation of a ferryl intermediate in the high spin state, along with the release of CO_2 and succinate. Then, the oxygen atom on the Fe center is reoriented, and demethylation happens to produce the undamaged base and formaldehyde (Cisneros 2010). MD simulations further note that D135 is an important residue in AlkB, participating in both substrate specificity and catalysis for adenine and cytosine substrates through favorable interactions with the exocyclic amines of the bases, whereas the oxygen atoms at the 6 position of guanine and 4 position of thymine are subject to electrostatic clashes (Holland and Hollis 2010).

Until now, nine homologs of AlkB (ALKBH1–8 and FTO) and TET proteins (TET1–3) have been identified as the major 2-OG/Fe(II) oxygenases to catalyze biological oxidation of DNA/RNA biomarkers in human cells (Tables 1, 2 and 3) (Fedeles et al. 2015). ALKBH1 was the first mammalian AlkB homolog to be identified in 1996, primarily localized in the mitochondria or the nucleus in certain cell lines (Wei et al. 1996; Haag et al. 2016; Xie et al. 2018). As reported, substrates of ALKBH1 are different types of methylated nucleotides of DNA or RNA, methylated lysine of histone H2A, and abasic sites of DNA (Müller et al. 2010; Ougland et al. 2012). A lack of ALKBH1 leads to sex-ratio distortion, unilateral eye defects,

Table 1 DNA/RNA biomarkers modified by 2-OG/Fe(II)-dependent oxygenases

Biomarker	2-OG/Fe(II)-dependent oxygenase
m1A	DNA: AlkB, ALKBH2, ALKBH3 RNA: AlkB, ALKBH3, ALKBH7
m3C	DNA: AlkB, ALKBH1, ALKBH2, ALKBH3 RNA: AlkB, ALKBH1, ALKBH3
m1G	DNA: AlkB, ALKBH2 RNA: AlkB
m3T	DNA: AlkB, ALKBH2, ALKBH3, FTO RNA: FTO
m4C	DNA: AlkB
m2G	DNA: AlkB
O6mG	DNA: mutant AlkB (D135R, D135G, and D135A)
m5C	DNA: AlkB, ALKBH2, ALKBH3, TET1–3 RNA: ALKBH1, TET1–3
m6A	DNA: ALKBH1, ALKBH4 RNA: ALKBH3, ALKBH5, FTO
T/base J formation	DNA: TET1–3/JBP1 and 2
e1A	DNA: AlkB, ALKBH2, ALKBH3
N^3-EtdT	DNA: ALKBH2, ALKBH3
εA	DNA: AlkB, ALKBH2
εC	DNA: AlkB, ALKBH2, ALKBH3
$1,N^2$-εG	DNA: AlkB, ALKBH2
m_2^2G	RNA: mutant AlkB (D135S/L118V), ALKBH7
mc5mU	RNA: ALKBH8
e2G, EA, FF, HF, αHOPG, γHOPG, M_1dG, HEC, HPC	AlkB

and impaired placental trophoblast lineage differentiation in mice (Pan et al. 2008; Nordstrand et al. 2010).

ALKBH2 is located in the nucleus and linked to human bladder and lung cancers (Fujii et al. 2013). This DNA demethylase usually prefers a double-stranded nucleotide substrate. The crystal structure of ALKBH2 binding to ds-DNA indicates that ALKBH2 uses Phe 102, an aromatic finger residue, to intercalate into the duplex stack and recognize the damaged base (Yang et al. 2008).

ALKBH3 is mainly located in the cytoplasm and in the nucleus (Tsujikawa et al. 2007). As reported, it is not only a DNA demethylase but also a tRNA demethylase. It contains 286 amino acids and is composed of NRL and DSBH domains. A β4-β5 hairpin creates a lid over the active site, which is necessary for DNA/RNA binding. In addition, His191, Asp193, and His257 are the active site residues for iron binding. Moreover, the substrate-binding pocket of ALKBH3 is considerably more polar than that in AlkB or ALKBH2, which may explain the narrow substrate scope of ALKBH3 (Sundheim et al. 2006).

Table 2 2-OG/Fe(II)-dependent oxygenases, DNA/RNA substrates, and representative crystal structures

Enzyme	Protein Data Bank (PDB) code	Substrate
AlkB	4NID (m6A) 3KHC (m1G) 3KHB (Co/2-OG) 3I3Q (Mn/2-OG) 3BI3 (m1A/cofactors) 3BIE (m1A/Mn/2-OG) 3BKZ (DNA/active site)	DNA: m1A, m3C, m1G, m3T, m4C, m2G, O6mG (mutant AlkB D135R, D135G and D135A), m5C, e1A, εA, *εC, 1,N^2-εG,* e2G, EA, FF, HF, *α*HOPG, *γ*HOPG, M1G, HEC, HPC
		RNA: m1A, m3C, m1G, m_2^2G (mutant AlkB D135S/L118V)
ALKBH1	6 IE2, 6 IE3 6KSF (DNA bulge)	DNA: m3C, m6A
		RNA: m3C, m5C
ALKBH2	3S5A (DNA/cofactors) 3BUC (Mn/2-OG) 3BTX (DNA) 3BTY (m1A) 3BTX (DNA/active site)	DNA: m1A, m3C, m1G, m3T, m5C, e1A, N^3-EtdT, εA, *εC, 1,N^2-εG*
ALKBH3	2IUW (Fe/2-OG)	DNA: m1A, m3C, m3T, m5C, e1A, N^3-EtdT, *εC*
		RNA: m1A, m3C, m6A
ALKBH4	–	DNA: m6A
ALKBH5	4NJ4 4NRO (2-OG) 4OCT (Mn/2-OG)	RNA: m6A
ALKBH6	–	–
ALKBH7	4QKD (Mn/2-OG)	RNA: m1A, m_2^2G
ALKBH8	3THT	RNA: mc5mU
FTO	3LFM 5ZMD (m6A)	DNA: m3T
		RNA: m3T, m6A
TET1–3	4NM6 (TET2-DNA) 5D9Y (TET2-f5C) 5DEU (TET2-h5mC) 5EXH (TET3-ca5C)	DNA: m5C, T
		RNA: m5C
JBP1–2	2XSE (JBP1-DNA)	DNA: T

ALKBH4 is an enzyme existing in the cytoplasm and the nucleus and is related to lethal embryonic disorders (Nilsen et al. 2014). This enzyme has the ability to demethylate m6A in DNA and also mediates demethylation of a monomethylated site in actin (K84me1), which regulates the actin-myosin interaction and other

Table 3 Kinetic parameters of 2-OG/Fe(II)-dependent oxygenases on alkyl biomarkers

Enzyme	Substrate	k_{cat} min^{-1}	*K*m μM	k_{cat}/Km $min^{-1} \cdot \mu M^{-1}$
AlkB	ss-m1A	4.2 ± 0.2	7.1 ± 1.1	0.59 (Chen et al. 2017)
	ds-m1A	4.8 ± 0.2	12.7 ± 1.3	0.38 (Chen et al. 2017)
	ss-m3C	24.5 ± 0.7	19.9 ± 1.3	1.23 (Chen et al. 2017)
	ds-m3C	8.2 ± 0.4	10.8 ± 1.9	0.76 (Chen et al. 2017)
	TεAT	0.13 ± 0.05	60 ± 14	0.002 (Yu and Hunt 2009)
	m1G	0.052 ± 0.008	3.3 ± 1.3	0.016 (Wang et al. 2021)
ALKBH2	ss-m1A	1.1 ± 0.1	4.1 ± 0.9	0.28 (Chen et al. 2017)
	ds-m1A	2.5 ± 0.1	7.3 ± 0.9	0.34 (Chen et al. 2017)
	ss-m3C	1.7 ± 0.1	1.4 ± 0.2	1.21 (Chen et al. 2017)
	ds-m3C	2.6 ± 0.1	1.9 ± 0.4	1.34 (Chen et al. 2017)
ALKBH3	ss-m1A	1.2 ± 0.0	2.3 ± 0.1	0.51 (Chen et al. 2017)
	ss-m3C	1.7 ± 0.1	1.9 ± 0.4	0.87 (Chen et al. 2017)
TET2	m5C			0.27 (Hu et al. 2015)
	hm5C			0.042 (Hu et al. 2015)
	f5C			0.021 (Hu et al. 2015)
ALKBH1	Bulged m6A			0.043 (Zhang et al. 2020)
	ss-DNA			0.028 (Zhang et al. 2020)
FTO	m3U			0.014 (Jia et al. 2008)
	m3T			0.007 (Jia et al. 2008)

actomyosin-dependent processes, such as cytokinesis and cell migration (Li et al. 2013b).

ALKBH5 localizes predominantly to the nuclear speckles where its demethylation activity may influence mRNA processing and mouse fertility (Zheng et al. 2013). ALKBH5 is known to demethylate m6A in RNA and prefer single-stranded to double-stranded substrates, consistent with the role as an RNA demethylase.

ALKBH6 in humans is localized mainly in the nucleus and in the cytoplasm (Tsujikawa et al. 2007; Mielecki et al. 2012). ALKBH6 has been reported to play an important role in seed germination, seedling growth, and survival of *Arabidopsis* under abiotic stresses (Huong et al. 2020), and it disrupts the MMS-induced cytotoxicity seen in pancreatic cancer (Zhao et al. 2021). However, the substrate of ALKBH6 has yet to be discovered.

ALKBH7 is nuclear encoded but is predominantly localized in the mitochondria of mammalian cells (Zhang et al. 2021). ALKBH7 demethylates m_2^2G and m1A within mitochondrial Ile and Leu1 pre-tRNA regions. Furthermore, it may regulate the processing and structural dynamics of polycistronic mitochondrial RNAs, and it also plays a role in alkylation-induced necrosis and obesity (Fu et al. 2013a; Solberg et al. 2013; Zhang et al. 2021).

ALKBH8 is exclusively located in the cytoplasm and has three domains: the N-terminal RNA recognition motif, the middle 2-OG/Fe(II)-dependent AlkB domain, and the C-terminal methyltransferase domain (Pastore et al. 2012). Its

structure and hydroxylation of mcm5U (Fig. 1) suggest a potential role in the regulation of posttranscriptional tRNA modification through methylation/demethylation.

FTO was first discovered in a fused-toe mutant mouse (Peters et al. 1999). It is primarily expressed in the brain and adipose tissues (Gerken et al. 2007; Zheng et al. 2013). m6A in nuclear RNA is proposed to be the major substrate of FTO, supported by its partial localization in nuclear speckles (Jia et al. 2011). FTO prefers single-stranded to double-stranded substrates. Crystallographic studies demonstrate that FTO possesses an extra loop that competes with an unmethylated strand of DNA/RNA duplex for binding to FTO (Han et al. 2010).

TET1 and TET2 are primarily localized in the nucleus, whereas TET3 is localized in both the cytoplasm and the nucleus (Arioka et al. 2012). TET proteins are large multi-domain dioxygenases that contain a catalytic C-terminal domain. The catalytic domain comprises a cysteine-rich domain, a conserved double-stranded β-helix domain, and binding sites for the cofactors Fe(II) and 2-OG (Rasmussen and Helin 2016). In addition to their catalytic domain, the mammalian TET subfamily members, TET1 and TET3, have an N-terminal CXXC domain that can recognize clustered unmethylated CpG sequences (Lu et al. 2015). In contrast, TET2 typically shows a substrate preference for m5C in a CpG sequence (Hu et al. 2013). A crystal structure of TET2 reveals three zinc cations in the cys-rich domain, two of which are coordinated by TET2 residues and can stabilize critical parts of the enzyme's tertiary structure for catalysis and DNA interaction (Lu et al. 2015).

JBP1 and JBP2 proteins were characterized to be the first members of the TET/JBP protein family from *Trypanosoma* and *Leishmania* (Cliffe et al. 2010). Both JBP1 and JBP2 catalyze the hydroxylation of the methyl group in thymine. They are key regulatory enzymes of base J-synthesis (Fig. 1) (Cliffe et al. 2009). JBP2 is a chromatin remodeling protein that is critical for de novo J-synthesis, whereas JBP1 can bind base J in ds-DNA to stimulate additional J-synthesis. Sequence analysis of the JBP hydroxylase domains revealed that JBP proteins belong to the class of 2-OG/Fe(II)-dependent dioxygenases, and they are *kinetoplastid* homologs of the TET proteins (Iyer et al. 2009; Cliffe et al. 2012).

Brief Summary of Spectroscopic and Computational Studies

Spectroscopic and computational studies on the abovementioned proteins have been extensively carried out in line with biochemical studies, providing further insights into their interplay and mechanisms of action (Mishina et al. 2004; Bleijlevens et al. 2007; Proshlyakov et al. 2017). EPR spectroscopy is a powerful technique to obtain structural information about the active site of 2-OG/Fe(II)-dependent oxygenases. Through EPR and UV-visible spectroscopic studies on the Fe binding environment of the proposed catalytic center of wild-type ALKBH4, a novel, unusual high-spin Fe(III) species was observed by EPR in the presence of sulfide, with a g_{max} of 8.2. These studies suggest that an intact histidine-carboxylate site is necessary for productive Fe binding (Bjørnstad

et al. 2011, p. 4). Indeed, there is no change in the EPR spectrum during binding of methylated DNA to the AlkB/Cu/2-OG complex, demonstrating that the substrate does not bind in the immediate vicinity of the metal center (Bleijlevens et al. 2007).

Molecular dynamics (MD) and combined quantum mechanics/molecular mechanics (QM/MM) methods have proven useful to investigate how structural dynamics influence the selectivity and mechanism of 2-OG/Fe(II)-dependent DNA/RNA-modifying enzymes (Quesne et al. 2014; Wang et al. 2015; Liu et al. 2017; Torabifard and Cisneros 2017; DeNizio et al. 2019; Waheed et al. 2020, 2021; Ramanan et al. 2021). Those methods have been used to explain why N^2,3-εG escapes AlkB-mediated repair. The disrupted π–π interactions lead to a large oxidation barrier when AlkB or ALKBH2 binds N^2,3-εG (Lenz et al. 2020). In addition, the calculations reveal that 2-OG rearrangement prior to binding of O_2 to the active site Fe is preferred to a ferryl rearrangement to generate the catalytically active Fe (IV) = O intermediate in AlkB and ALKBH2 (Waheed et al. 2020). Hydrogen atom transfer proceeds via a σ-channel in ds-DNA (m3C), while there is a competition between σ- and π-channels in ss-DNA (m3C), implying that DNA biomarkers have the potential to alter molecular orbital interactions during the demethylation process (Waheed et al. 2020). The studies explored the role of the long-range correlated motions to facilitate the substrate binding and catalysis in AlkB, ALKBH2, and TET2 enzymes (Waheed et al. 2020, 2021). Computational methods have been applied to explore the effect of clinically important mutations on the structure and dynamics of TET2 enzyme-substrate complex, the reaction pathway and orbital mechanism of the HAT reaction, and its kinetic isotope effects (Waheed et al. 2021).

.Overall the computational studies reveal the specific mechanistic strategies of the 2-OG/Fe(II) nucleic acid modifying enzymes, the complex dynamics of their enzyme-substrate complexes, the multifaceted correlation between the enzymes and substrates, the catalytic mechanisms, and the atomistic effects of mutations related to diseases.

Applications to Prognoses, Other Diseases, or Conditions

Cell toxicity is caused by certain exogenous toxicants, such as chemical agents, environmental pollutants, and pharmaceutical drugs, each of which can damage the cell and contribute to serious dysfunction. The mechanism of cell toxicity has been linked to overproduction of nitric oxide (NO), reactive oxygen species (ROS), and subsequent oxidative stress (Beckman and Koppenol 1996). Toxicants may also induce and release compounds that directly damage DNA/RNA, causing cell apoptosis and toxicity.

DNA/RNA methylation is a common phenomenon in genetic/epigenetic modification in humans (Gibney and Nolan 2010). The methylation level has a considerable effect on cell division, differentiation, apoptosis, and aging. Myriad human diseases result from hypermethylation on DNA promoter regions. As such, methylation levels may prove useful in the early detection of human diseases (Levenson 2010). Indeed, hypermethylation of promoter regions in *APC*, *RASSF1A,* and TP53 genes are considered as biomarkers for an early cancer diagnosis. Moreover,

dysregulation of miRNAs has been observed in breast cancer, which has the potential to be used in the early cancer evaluation (van Schooneveld et al. 2015). Various studies have revealed that genome-wide DNA methylation alterations are linked to aging and age-related diseases. It is well established that the biological age of certain tissue types can be predicted through DNA biomarkers across the entire human lifespan (Salameh et al. 2020). Furthermore, recent research provides compelling evidence of DNA methylation associated with neurodegenerative diseases, autoimmune disorders, and autoimmune disorders (Moosavi and Motevalizadeh Ardekani 2016). The correlation between genetic/epigenetic biomarkers and specific diseases will further help to develop advanced diagnostic techniques for these diseases and to prevent them.

Conclusions and Perspectives

DNA/RNA biomarkers (Fig. 2) are ubiquitous in cells and tissues. The study of the biochemical mechanism of 2-OG/Fe(II)-dependent enzymes (Fig. 1) is very helpful to connect exogenous or endogenous factors to human health. Since the characterization of DNA/RNA biomarkers and the identification of the AlkB family and TET/JBP proteins, an increasing number of studies have been carried out to explore the potential relation between direct reversal repair and biological regulations. In vivo and in vitro studies have demonstrated the function of many enzymes related to specific biomarkers. However, fundamental questions remain unanswered. The substrate of ALKBH6 is still unclear. Systematic studies need to be conducted to determine whether the enzymes discussed in this chapter have the potential to modify other unreported DNA/RNA biomarkers. m6A and m5C, as well as their oxidative intermediates, have been crucial in epigenetic regulation, such as transcriptional regulation, gene silencing, and reprogramming, but the mechanisms and pathways of these epigenetic markers and whether epigenetic alterations could serve as a prognostic marker in disease diagnoses still need to be carefully evaluated (Pastor et al. 2011; Cimmino and Aifantis 2017; Klungland and Robertson 2017). Overexpression of AlkB family proteins in some cancers, such as prostate, bladder, and pancreatic cancers, can promote tumor cell growth or chemotherapy resistance (Shimada et al. 2009; Koike et al. 2012). Therefore, it will be valuable to carry out further studies on whether 2-OG/Fe(II)-dependent oxygenases can serve as potential targets in drug development, clinical therapy, and toxicology (Table 3).

Mini-Dictionary of Terms

- *Direct reversal repair: It is a type of DNA repair where the modified area, mainly DNA bases, is repaired directly by specialized enzymes but not breaking the DNA strand.*
- *Lipid peroxidation: It is a chain reaction and created by free radicals influencing unsaturated fatty acids in cell membranes, leading to oxidative damage.*

- *Base excision repair: A type of DNA repair where a modified base is excised by a DNA glycosylase to generate an abasic site, followed by excision of the resulting sugar phosphate. The generated gap in the DNA helix is then filled in by the sequential action of DNA polymerase and DNA ligase.*
- *Adaptive response: It is a form of direct DNA repair in E. coli that protects DNA from alkyl damage, including four genes (ada, alkA, alkB, and aidB).*

Key Facts of 2-Oxoglutarate/Fe(II)-Dependent Oxygenases

2-Oxoglutarate/Fe(II)-dependent dioxygenases are a superfamily of enzymes that play diverse roles in biological process. In bacteria, AlkB is associated with DNA repair. In mammalian cells, enzymes (ALKBH1–8, FTO, and TET/JBP) have functions in genome protection, epigenetic regulation, biosynthesis, and posttranslational modification. Mechanistically, 2-OG-dependent dioxygenases catalyze oxidative reaction by inserting a single oxygen atom to form molecular oxygen into the substrate. The co-substrate 2-OG will share the other oxygen atom to generate succinate and carbon dioxide.

Summary Points

- *Genetic and epigenetic biomarkers, particularly the alkylated DNA/RNA, are prevalent in cells and tissues, providing potential applications to prognoses, other diseases, and conditions.*
- *DNA/RNA alkylation, particularly methylation, usually results from endogenous and exogenous agents.*
- *AlkB family (E. coli AlkB and its mammalian homologs ALKBH1–8 and FTO) and TET family (TET1–3/JBP) play important roles in dealkylation/oxidative modification of DNA/RNA biomarkers. 2-OG/Fe(II)-dependent oxygenases employ Fe(II) as a cofactor and 2-OG and O_2 as co-substrates with release of CO_2 and succinate during catalysis.*
- *Spectroscopic and computational studies provide further insights into how structural dynamics influence the selectivity and mechanism of 2-OG/Fe(II)-dependent DNA/RNA-modifying enzymes.*

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DNA Adducts as Biomarkers in Toxicology 17

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Abstract

The measurement of biopolymer modifications due to exposure to environmental chemicals or endogenous physiological processes can provide valuable information on toxicological exposure and disease risk. The covalent modification of DNA represents an especially important case since unrepaired DNA adducts can have wide-ranging genetic and epigenetic consequences with significant impact on human health. In this chapter, we examine how quantification of DNA adducts can inform on exposure to a variety of DNA damaging agents present in the environment and those produced endogenously due to physiological processes. This information can be used to set limits on exposure and for predicting susceptibility to genetic diseases such as cancer, diabetes, and immunological dysfunction. With the development of increasingly sensitive analytical methods that also provide detailed structural information, we are on the verge of a new era of DNA adductomics – the complete characterization of the entire spectrum of DNA damage in humans. In this review, we will provide a brief overview of the various methods that have been used to measure DNA adducts as well as a critical appraisal of their strengths and weaknesses. We will then highlight some of the more important DNA adducts that have been linked to human disease and conclude with some examples of how measurement of chemotherapy induced adducts may be used to predict treatment response.

Keywords

DNA adducts · Biomarkers · Environment · Toxicants

Abbreviations

1,N^6-HMHP-dA	1,N^6-(1-hydroxymethyl-2-hydroxypropan-1,3-diyl)-2′-deoxyadenosine
6–4 PP	(6–4) pyrimidine/pyrimidone photoproducts
8-MEIQx	2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline

AαC	2-amino-9H-pyridole[2,3-b]indole
ALDH2	Alcohol dehydrogenase 2
AMS	Accelerator mass spectrometry
BaP	Benzo[α]pyrene
BD	1-3-Butadiene
bis-*N7*G-BD	1,4-bis-(guan-7-yl)-2,3-butanediol
BPDE	trans-7,8-dihydroxy-anti-9,10-epoxy-7,8,9,10-tetra-hydrobenzo[a]pyrene
CEdG	N^2-Carboxyethyl-2′-deoxyguanosine
CPDs	Cyclopyrimidine dimers
DEB	1,2,3,4-diepoxybutane
EB	3,4-epoxy-1-butene
EBD	1,2-dihydroxy-3,4-epoxybutane
EB-GI	*N7*-(2-hydroxy-3-buten-1-yl)-guanine
EB-GII	*N7*-(1-hydroxy-3-buten-2-yl)-guanine
HAAs	Heterocyclic aromatic amines
IQ	Amino-3-methylimidazo[4,5-f]quinoline
IR	Ionizing radiation
LC-MS/MS	Liquid chromatography tandem mass spectrometry
MelQ	2-amino-3,4-dimethylimidazo[4,5-f]quinoline
MeIQx	2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
MG	Methylglyoxal
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
N1-THB-Ade	*N1*-(2,3,4-trihydroxybut-1-yl)-adenine
N^2-ethylidene-dGuo	N^2 -ethylidenedeoxyguanosine
N^2-HOMe-dG	N^2- hydroxymethyl-dG
N^6-HOMe-dA	N^6-hydroxymethyl-dA
N7-MedG	*N7*-methyldeoxyguanosine
N7-THBG	*N7*-(2,3,4-trihydroxybut-1-yl)-guanine
NOCs	N-nitroso compounds
NNK	4-(methylnitrosamino-1-(3-pyridyl-butanone
NNN	*N*-nitrosonornicotine
O^6-MedG	O^6-methyldeoxyguanosine
PAHs	Polycyclic aromatic hydrocarbons
PDG	α-CH_3-γ-OH-1, N^2-propano-2′-deoxyguanosine
PhIP	2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
SRM	Selective reaction monitoring

Introduction

The human genome is under constant assault, with ~10^5 DNA lesions per cell induced from a variety of endogenous and exogenous sources (Lindahl 1993). Despite overlapping repair pathways, histone protection, and a highly condensed

structure, exogenous chemicals can bypass these barriers causing persistent DNA modifications. As opposed to the rapid turnover of modified protein and lipids, DNA adducts can have long-lasting impacts if left unrepaired. Modified DNA contributes to disease etiology by interfering with DNA regulatory protein binding, inducing mutations, promoting transcriptome miscoding, and precipitating strand breaks due to replication fork collapse by polymerase-blocking lesions.

Endogenous DNA adducts can serve as biomarkers for disease (e.g., chronic inflammation; metabolic syndrome), whereas adducts formed by exogenous agents can provide information on toxicological exposure and environmental risk assessment. Continual refinement of analytical methods for DNA adduct measurement, particularly mass spectrometry (MS), has made it possible to comprehensively characterize all detectable DNA adducts – the DNA adductome. This effort will contribute significantly to our understanding of how environmental exposures and endogenous DNA damage contribute to cancer, diabetes, immunological dysfunction, and a host of other diseases.

While mass spectrometric methods are rapidly becoming the standard in the field, most of the literature is based on a variety of methods with varying levels of sensitivity and ability to unambiguously characterize structure. It is often difficult if not impossible to compare results obtained across different platforms. Nonetheless, it is important to understand different approaches so that the benefits and limitations of each can be appreciated. For example, some antibody-based methods may offer superior sensitivity for detection, but the potential for cross-reactivity with nontargeted antigens can confound interpretation of results. What follows is a brief introduction to methods commonly used to quantify DNA adducts for biomonitoring.

^{32}P Postlabeling

The postlabeling method was developed in the 1980s and has undergone several revisions to improve sensitivity and reproducibility (Phillips and Arlt 2007). It is still relatively popular owing to its sensitivity, simplicity, and low cost. Modified DNA is first enzymatically digested to 3′-monophosphates and then 5′-labeled with ^{32}P prior to elution in two dimensions on polyethylene imine cellulose. The modified nucleotides elute along the diagonal and have vastly different elution properties relative to unmodified nucleotides. While there are some general guidelines for choosing solvent systems, this needs to be optimized for each adduct of interest. Following the initial digestion step, DNA adducts are enriched over unmodified nucleotides, and with these additional steps, sensitivity as low as ~$1/10^{10}$ nt can be attained. A commonly employed enrichment method is a second enzymatic digestion with nuclease P1, which removes 3′ phosphates. While many DNA adducts are resistant to P1 digestion, unmodified nucleotides are readily dephosphorylated. As a 3′ phosphate is necessary for 5′-OH labeling by ^{32}P with polynucleotide kinase, this extra step eliminates the major fraction of non-adducted nucleosides and enriches DNA adducts. This approach increases sensitivity by as much as 1 in 10^{10} nt. However, not all DNA adducts are resistant to P1, and some useful guidelines

have been established (Gupta and Earley 1988). Other approaches for adduct enrichment include antibody affinity columns, solvent extraction, and liquid chromatography.

The main drawback of the postlabeling method is the lack of structural verification. Authentic standards facilitate identification and allow quantification of DNA adducts. However, even with well-resolved chromatograms and authentic standards, positive identification can be ambiguous. Yet due to its sensitivity and the fact that it can be performed without the need for sophisticated equipment, postlabeling is still used in DNA adduct studies.

Antibody-Based Methods

Antibody-based approaches are the most widely used methods for DNA adduct detection and quantification (Santella 1999). Because DNA adducts are generally not sufficient to provoke an antibody response, adducts must be presented on a protein. This can be accomplished non-covalently by complexing adduct-containing DNA to a methylated carrier protein or by covalent attachment of the monoadduct of interest to keyhole limpet hemocyanin or BSA. The noncovalent method requires a high adduct density (~1 adduct/100 nt) to elicit an antibody response, whereas the latter approach is preferred for applications such as immunoaffinity chromatography. Polyclonal or monoclonal antibodies possess similar specificities, and many antibodies to a variety of DNA adducts are commercially available. For immunological quantification of DNA adducts, ELISA has largely replaced RIA. Antibodies with the highest affinities can quantify 1 adduct/10^8 nucleotides from 50 to 100 μg of DNA, with increased sensitivity possible with greater sample size. Immunohistochemistry can be used on fresh, frozen, or paraffin-embedded tissues with immunofluorescence or immunoperoxidase for qualitative/quantitative measurement. Even with the increased availability of MS approaches which afford more precise quantification and structural verification, antibody-based approaches still possess great utility. Immunoaffinity purification prior to analysis by HPLC or LC-MS enhances the detection of rare adducts by reducing unwanted analytes and reducing matrix effects from biological samples. For example, the malondialdehyde-derived DNA adduct, 6-oxo-M1dG, was shown to be eliminated predominantly through feces (350–1893 fmol/kg/day) using a sepharose-based affinity column pre-purification prior to LC-MS/MS with isotope dilution (Akingbade et al. 2012).

The major confounding issue surrounding antibody-based approaches for DNA adduct measurement is cross-reactivity. For example, antibodies produced by immunization with 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydro-benzo(a)pyrene (BPDE) cross-react with many polyaromatic hydrocarbons (PAHs). Antibodies that recognize DNA adducts can also cross-react with RNA analogs, e.g., antibodies for the major DNA adduct of methylglyoxal. N^2-Carboxyethyl-2′-deoxyguanosine (CEdG) also react with CEG in RNA (Schneider et al. 2004). Whenever possible, antibody measurements should be calibrated by quantitative methods, preferably by MS.

MS Methods

MS coupled with high-resolution chromatography is the preferred method for DNA adduct detection and measurement. While sensitivity is generally less than ^{32}P postlabeling, acquisition of detailed structural information simultaneously with quantification for multiple adducts has allowed for major advances in adductomics (Balbo et al. 2014; Guo et al. 2017; Rappaport et al. 2012). Adducts are quantified by fitting data to a standard curve generated with increasing amounts of authentic standards spiked with a fixed amount of isotopically enriched standards, containing 3–5 ^{15}N or ^{13}C substituted atoms. Several MS approaches have been used to measure DNA adducts, with varying levels of sensitivity and resolution. While an in-depth discussion of MS methods for DNA adduct measurement is beyond the scope of this review, the most common approaches will be briefly discussed, and their advantages and shortcomings are presented to allow for a critical evaluation of the literature in this field. For a more detailed MS introduction, the reader is referred to these excellent monographs: MS: Principles and Applications, Edmond de Hoffmann and Vincent Stroobant, third Edition, 2007, John Wiley and Sons, Ltd. and Introduction to Mass Spectrometry, J. Throck Watson and O. David Sparkman, fourth Edition, 2007, John Wiley and Sons, Ltd.

Accelerator MS (AMS)

One of the most sensitive approaches for the detection of DNA adducts is AMS, which has a limit of detection (LOD) of one adduct/10^{12} nucleotides (Enright et al. 2016). Attomolar (10^{-18} mol) sensitivity is possible using isotopically enriched standards, typically ^{14}C and measuring the $^{14}C/^{13}C$ isotopic ratio. The method's extreme sensitivity allows administration of very low doses of radioactivity (microdosing) without significant risk of adverse effects, making it a useful method for pharmacokinetic or drug metabolism studies. Following administration of the isotopically labeled compound of interest, tissues or biological matrices are converted by high temperature into CO_2, with further reduction to graphite in the "traditional" method of analysis (Ognibene et al. 2003) prior to isotope ratio measurements. A gas accepting ion source enables direct CO_2 analysis without conversion to graphite, offering advantages of higher throughput, smaller sample size, and greater sensitivity (Ognibene et al. 2015). As the analyte is destroyed during the AMS measurement process, no structural information is obtained. AMS sensitivity makes it feasible to study the distribution of environmental toxins at real-world exposure levels.

Gas Chromatography-MS (GC-MS)

Many early MS studies used GC-MS to identify and quantify DNA adducts (Rehman et al. 2000). This requires derivatization of polar DNA adducts to convert them into volatile species that can be eluted from the GC column interface into the

ionization chamber. DNA samples are acid-hydrolyzed at 150 °C to cleave the phosphodiester backbone and release free bases prior to derivatization by trimethylsilylation to render them volatile (Aruoma et al. 1989). The samples are then introduced into the injection port at 250 °C. Chromatographic separations are accomplished using fused silica capillary columns with helium as a carrier gas with temperature gradients from 125 °C to 290 °C. To avoid artifact formation, analytes must be thermally stable, and reactions must exclude oxygen as oxidized derivatives are produced at elevated temperatures. In 1997, the European Standards Committee on Oxidative DNA Damage (ESCODD) distributed oligonucleotide samples containing a known amount of 8-OHdG to 25 laboratories across Europe and compared results obtained using GC-MS, LC-MS, and HPLC-EC (electrochemical detection) (Riis 2002). GC-MS analyses consistently overestimated 8-OHdG levels, whereas LC-MS and EC yielded results within 53% and 73% of expected values, respectively.

LC-MS/MS

The ability to fragment and identify ions using tandem mass spectrometry (MS/MS) has led to rapid advances in DNA adduct biomonitoring. The most common in this application is the utilization of spectrometers with three linear quadrupoles interfaced with an electrospray source preceded by HPLC or UHPLC. The first and third quadrupoles (Q1 and Q3) detect ions and function as true mass spectrometers, whereas the middle functions as a collision cell and is an RF-only quadrupole (q). A carrier gas, typically helium, is used to fragment ions either selected or scanned in Q1, which are then detected in Q3. Different scan modes may be utilized to identify and quantify DNA adducts. Ions with a predetermined *m/z* are selected in Q1, fragmented in q, and scanned over a range of *m/z* in Q3 (product ion scan). Alternatively, ions may be scanned over a mass range in Q1, and a specific fragment ion detected in Q3 (parent ion scan). In a third configuration, both Q1 and Q3 scan a mass range, but with an offset between the two mass spectrometers corresponding to loss of a specific ion X. Thus, for every ion m scanned in Q1, if loss of X occurs in the collision cell, m-X will be detected in Q3. This last modality is called constant neutral loss. Loss of the deoxyribose moiety (116 amu) is a common fragment for nucleic acid adducts, thus setting X to 116 is often used for DNA adduct identification.

As the geometry of the triple quadrupole only allows analysis of ions resulting from a single collision event (MS^2), approaches have been designed to allow for higher-order ion fragmentation (MS^{2+n}) to facilitate structural analysis. This is possible using ion trap instruments, which use an oscillating DC electric field to sequester ions in three dimensions, which are then selected for additional fragmentations. The most advanced and sensitive ion trap instrument is the electrostatic trap or Orbitrap, which sequesters ions in an intricate spiral trajectory around a central electrode using an oscillating electric field. The Orbitrap may be considered the current state-of-the-art instrument for DNA adduct monitoring and adductome characterization (Guo et al. 2017).

DNA Adducts as Biomarkers for Alcohol Consumption

It has been challenging to evaluate the impact of alcohol on human health, as levels of alcohol consumption are largely self-reported. Quantitative assays to measure alcohol intake would allow for more precise biomonitoring. Ethanol is oxidized to acetaldehyde by cytochrome P4502E1 (Cyp2E1). Acetaldehyde [**1**] is a highly reactive carbonyl which can either react with primary amines forming Schiff bases or undergo enolization and participate in aldol chemistry to yield crotonaldehyde [**2**] [Fig. 1, (Garcia et al. 2011; Theruvathu et al. 2005)]. It is thus important that

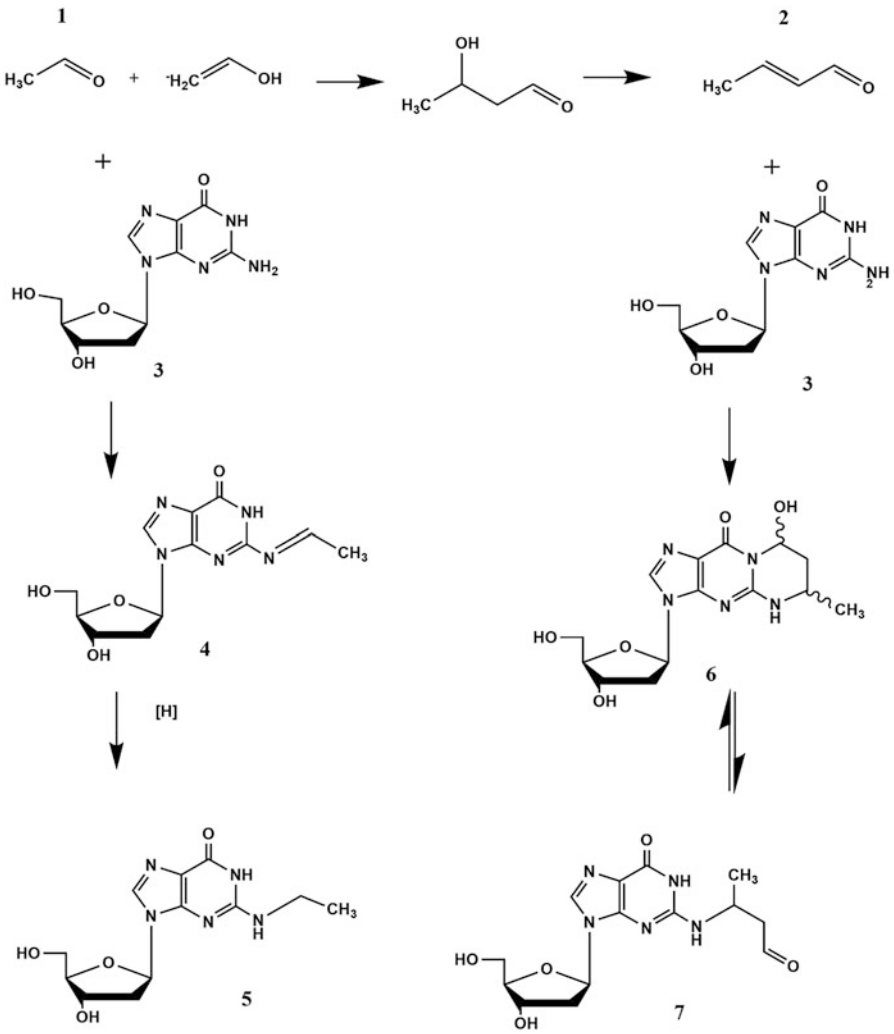

Fig. 1 Alcohol adducts

acetaldehyde be rapidly detoxified. This is accomplished by the cytosolic enzyme alcohol dehydrogenase 2 (ALDH2), which converts acetaldehyde to acetate with the production of NADH. Defects in ALDH2 expression result in increased acetaldehyde-associated pathological effects such as nausea, facial flushing, and tachycardia. Approximately 30% of individuals of East Asian decent are so afflicted, and acetaldehyde may contribute to their elevated incidence of esophageal cancer (Brooks et al. 2009; Yang et al. 2010; Yoshida et al. 1984). IARC accordingly classifies alcohol as a Group 1 carcinogen (Alcohol Consumption and Ethyl Carbamate. Volume 96, IARC 2011).

Acetaldehyde DNA Adducts

Acetaldehyde reacts at N^2 of guanine [**3**] to yield the Schiff base, N^2 -ethylidene-deoxyguanosine (N^2-ethylidene-dGuo, [**4**]). While [**4**] is relatively unstable with a half-life of ~24 h, it was measured in saliva of individuals exposed to alcohol using LC-ESI-MS/MS with selective reaction monitoring (SRM). Levels peaked 4 h after drinking and were ~ 100-fold over baseline, providing direct evidence for DNA adduct formation (Balbo et al. 2012). Evidence for the presence of [**4**] in human liver has also been presented (Wang et al. 2006). Because of its instability, biological studies on the repair and mutagenicity of [**4**] use the reduced form, N^2-ethyl-dG [**5**]. However, because of significant differences in H-bonding potential, stereochemistry, and hydrophobicity, it is unclear whether these studies provide an accurate assessment of the genotoxicity of [**4**]. Interestingly, [**5**] has been measured in humans using the ^{32}P postlabeling method in conjunction with HPLC/radioisotope detection and LC-ESI-MS-MS. N^2-ethyl-dG may result in vivo from biological reduction of the ethylidene derivative (Vaca et al. 1995). Postlabeling determined a level of 2–3 N^2-ethyl-dG adducts/10^7 nucleotides in DNA from a Swedish alcoholic cohort (Fang and Vaca 1997). Measurements of [**5**] in Japanese alcoholic patients who were either homozygous or heterozygous for the ALDH2* allele were found to have 28.3 and 3.9 N^2-ethyl-dG adducts/10^9 nucleotides, respectively, by LC-MS-MS (Matsuda et al. 2006). The significantly lower levels in the Japanese cohort relative to those determined for the Swedish patients may reflect in part differences in the analytical methods.

Crotonaldehyde DNA Adducts

Crotonaldehyde [**2**] reacts with 2′-deoxyguanosine forming α-CH_3-γ-OH-1, N^2-propano-2′-deoxyguanosine as a mixture of *R,S* stereoisomers (PdG [**6**]) in equilibrium with a ring opened form [**7**]. In double-stranded DNA, the equilibrium favors the open form, whereas the ring form predominates in single-stranded DNA. The open configuration [**7**] forms inter-strand cross-links when in a CpG sequence (Kozekov et al. 2003). Oligonucleotides containing PdG also cross-link peptides, suggesting that these adducts may disrupt protein-DNA interactions (Kurtz and

Lloyd 2003). In Japanese alcoholic patients homozygous for the ALDH2* allele, *S*-PdG and *R*-PdG levels were significantly higher relative to heterozygous individuals (92.4 ± 12.9 and 114 ± 15 fmol/μmol dG vs 42.9 ± 6 and 61.3 ± 6.4 fmol/μmol dG (Matsuda et al. 2006).

Radiation-Induced DNA Adducts

Ultraviolet light (UV) or ionizing radiation (IR) initiates oxidation reactions (loss of e^-) in DNA. Exposure to UV or IR, mitochondrial electron leakage creating ROS, and photosensitization by triplet sensitizers such as porphyrins to yield singlet oxygen ($^1O_2$) can all give rise to DNA adducts (Fig. 2). As guanine possesses the lowest ionization potential, it is the most frequent target of oxidative DNA damage. UV-induced DNA adducts are potential biomarkers of skin cancer, while those associated with IR are implicated in cancer promoting side effects of radiation therapy.

UV-Induced DNA Adducts

Prolonged sunlight exposure induces DNA adducts in a wavelength-dependent manner (Fig. 2). The main UV-induced DNA adducts are intra-strand cyclopyrimidine dimers (CPDs), predominantly in the *cis-syn* configuration (T-T **[8]**; T-C **[9]**), and (6–4) pyrimidine/pyrimidone photoproducts (6–4 PP **[10, 11]**). Early studies in UV-exposed human skin revealed CPDs in both the dermis and epidermis at 0.5 minimal erythema dose (MED) and 2.5 MED, respectively, by immunofluorescence (Eggset et al. 1983). A similar approach demonstrated CPDs in several skin tumor types including squamous cell carcinomas and actinic keratoses (Hori et al. 1992). Wavelengths of light in the UVA range (315–400 nm) comprise 75% of the solar spectrum. While these wavelengths are only negligibly absorbed by DNA ($\lambda_{max} = 280$ nm), they produce predominantly [**8**] in human skin as ascertained by both antibody and LC-MS/MS quantification (Mouret et al. 2006; Tewari et al. 2012). Thymine-cytosine [**9**] and the corresponding C-T dimers are also produced by UVA, in approximately tenfold lower-yield relative to [**8**]. No UVA-induced 6–4 PP have been detected in human skin. UVA also results in formation of 8-oxodG [**14**], consistent with O_2-mediated photosensitization. Quantification of UVA-induced T-T dimers by LC-MS/MS and 8-oxodG in human skin by HPLC-EC provided adduct values of 66 per 10^9 bases and 7.1 per 10^9 bases per J/m2, respectively (Mouret et al. 2006). The UVB portion of the solar spectrum (280–315 nm) is directly absorbed by DNA and produces a mixture of pyrimidine dimers and 6–4 PPs. Products induced by UVB measured by LC-MS/MS in decreasing order of abundance are [**8**] > [**9**] > [**11**] > C-T > C-C > [**10**]. [**8**] was produced in tenfold greater abundance than [**10**], while [**9**] was twofold more abundant than [**11**]. UVB produced twice as many T-T dimers relative to UVA. Absorption of a second photon by 6–4 PPs from either UVB or UVA promotes an intramolecular cyclization reaction yielding Dewar valence isomers [**12, 13**]. While initially thought to be of mechanistic interest only,

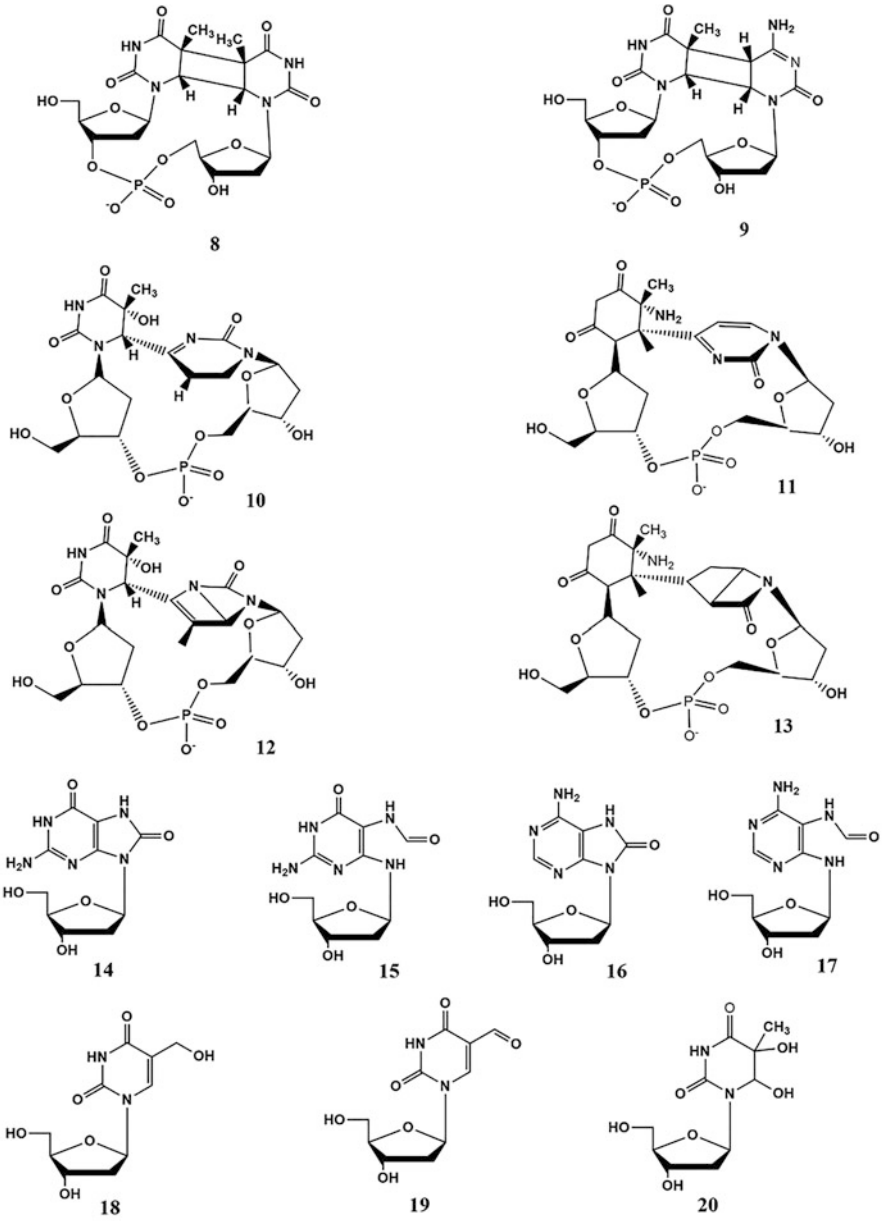

Fig. 2 UV and IR adducts

Dewar isomers are present in UVA/UVB-irradiated mouse skin and in cultured human mononuclear cells (Clingen et al. 1995; Qin et al. 1996). In human cells, Dewar isomers were the next most frequent adduct after pyrimidine dimers as

ascertained by antibody-based detection. Surface dwelling marine microorganisms from the Pacific Ocean contained significant basal levels of pyrimidine dimers, 6–4 PPs, and Dewar valence isomers by LC-MS-MS quantification (Meador et al. 2014). Adduct levels were similar between prokaryotic and eukaryotic organisms, with the majority (>80%) consisting of CPDs (~ 50% [**8**], 30% [**9**]), measured at a level of 15 CPDs/10^6 nucleotides at the equator. These results suggest that in addition to their potential etiologic role in skin cancer, UV-induced DNA adducts may have significant environmental impacts on ocean ecosystems.

IR-Induced DNA Adducts

DNA damage by IR occurs via reactions of the short-lived ($t_{1/2} \sim 10^{-9}$ s) hydroxyl radical (•OH) and direct formation of base radical ions. In the presence of oxygen, the latter intermediates can form peroxyl radical species and other base oxidation products such as hydroxyl [**18**] and formyl [**19**] derivatives (Martini and Termini 1997). Thus, the local O_2 concentration in tissue influences DNA adduct formation. In hypoxic tumors, low oxygen tension is largely responsible for resistance to therapeutic IR and provides the rationale for small molecule radiosensitizers which react with base radicals to induce cytotoxic DNA damage. Reaction of •OH with guanine yields the 8-hydroxyl-7,8-dihydroguanyl radical. Under oxidizing conditions, deprotonation, and loss of e-yields [**14**], whereas in the absence of oxygen, reduction of the dihydroguanyl radical produces FapydG [**15**] (Candeias and Steenken 2000). Analogous reactions occur at adenine to produce 8-oxodA [**16**] and FapydA [**17**], although with tenfold lower efficiency.

While more than 70 IR-induced DNA damage products have been described, the most biologically significant adducts are compiled in Table 1. The most abundant product is the saturated pyrimidine thymine glycol [**20**], generated as a mixture of four *cis* and *trans* isomers, arising from reaction of •OH with the 5,6 double bond. Single- and double-strand breaks, due to radical reactions at the phosphodiester backbone, as well as cross-linked products involving both ribose and the bases are also produced. While of biological importance, they are not usually used as biomarkers of IR exposure. DNA strand breaks increase linearly with IR dose and can be observed at doses as low as a mGy (Rothkamm and Löbrich 2003). IR is unique

Table 1 Ionizing radiation-induced DNA adducts[a]

Thymine glycol	**[20]**	97[b]
Fapy-dG	**[15]**	39
5-Hm-dU	**[18]**	29
5-formyl-dU	**[19]**	22
8-oxo-dG	**[14]**	20
Fapy-dA	**[17]**	5
8-oxo-dA	**[16]**	3

[a]Data adapted from (Cadet et al. 2008)
[b]#adducts/Gy/10^9 nt, by LC-MS/MS

among exogenous DNA-damaging agents in that it produces clustered sites of DNA damage, defined as two or more lesions within one or two helical turns (Lomax et al. 2013). These are challenging to repair and in conjunction with DNA strand breaks contribute to the high lethality of IR and therapeutic efficacy.

Heterocyclic Aromatic Amine (HAA) DNA Adducts

HAAs are produced from industrial manufacturing, tobacco combustion, cooked oils, and fried and grilled meats (IARC 2010). Some of these compounds are classified as established, probable, or possible human carcinogens. HAA levels are proportional to cooking time and temperature and can be as high as 500 ppb in well-done meats (Knize et al. 1994). The most abundant HAAs are 2-Amino-3,8-dimethylimidazo [4,5-f]quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) (Fig. 3) (Knize et al. 1994). Other HAAs, specifically 2-amino-9H-pyridole [2,3-b]indole (AαC), amino-3-methylimidazo[4,5-f]quinoline (IQ), and 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ), are at high hundreds ppb in beef extract (Matsumoto et al. 1981).

2-Amino-3,8-Dimethylimidazo[4,5-f] Quinoxaline (MeIQx)

MeIQx modifies dG to form dG-C8-MeIQx **[21]** (Fig. 3). Using ^{32}P postlabeling, **[21]** was detected in human colon and kidney at levels of 1 adduct/10^9 nucleotides (Totsuka et al. 1996).

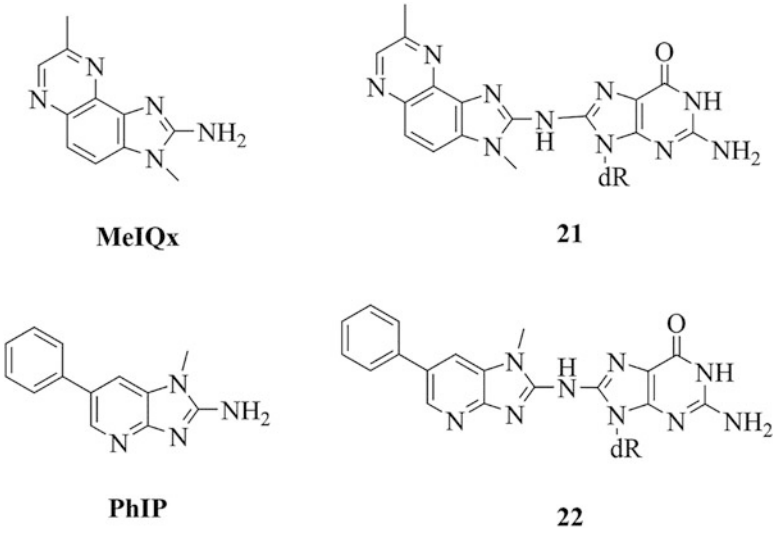

Fig. 3 Heterocyclic aromatic amines (HAAs)

2-Amino-1-Methyl-6-Phenylimidazo[4,5-b]Pyridine (PhIP)

PhIP is the most abundant HAA in well-done meat, and the presence of its DNA adducts increases the risk of colorectal, prostate, and breast cancers (Bouvard et al. 2015; Knize and Felton 2005; Turesky and Le Marchand 2011). The major PhIP-DNA adduct is dG-C9-PhIP **[22]**, measured at 2–120/10^9 nucleotides in human prostate samples (LC-MS/MS) (Fig. 3). Intriguingly, IHC determination yields considerably higher values (1/10^7 nucleotides), suggesting that antibody-based methods may have lower specificity for PhIP adducts than LC-MS/MS (Xiao et al. 2016). Elevated levels of PhIP adducts are associated with prostate cancer with an odds ratio of 3.4 ($p = 0.002$) (Zhu et al. 2006).

Aromatic Amine DNA Adducts

4-Aminobiphenyl (4-ABP)

4-ABP is found in diesel exhaust, tobacco smoke, and commercial hair dyes (Fig. 4) (Akyuz and Ata 2008; Davis and Bailey 1993; Luceri et al. 1993; Manabe et al. 1993; Turesky et al. 2003). Workers exposed to 4-ABP have an increased risk of bladder cancer (Collins et al. 1993; Collins et al. 1999). 4-ABP is metabolically activated to N-hydroxy-4-acetylaminobiphenyl (N-OH-ABP), which modifies DNA-forming *N*-(2′-deoxyguanosin-8-yl)-4-ABP (dG-C8–4-ABP, **[23]**), detectable

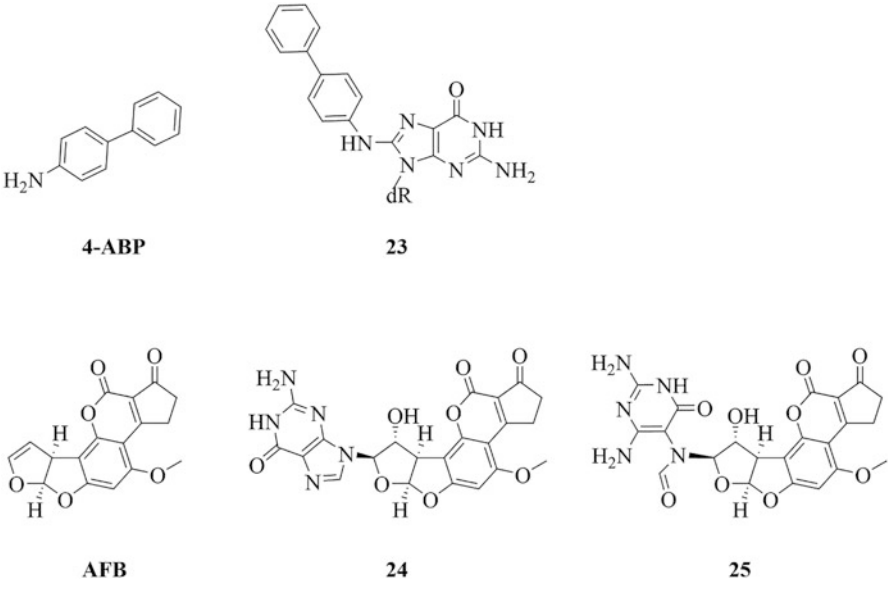

Fig. 4 Aromatic amines and aflatoxin adducts

in the bladder (Fig. 4) (Beland et al. 1983; Talaska et al. 1991). The reported MS LOD for **[23]** is 2 adducts/10^9 bases, and in human samples, **[23]** was present at 23 adducts/10^9 bases in nontumor tissue and 36 adducts/10^9 bases in bladder tumors (Zayas et al. 2007).

Aflatoxin B (AFB) DNA Adducts

Aflatoxins are environmental toxins and carcinogens discovered as causative agents in the deaths of fowls fed with diets containing peanut meal from South America (Blount 1961). Toxicity was associated with the presence of metabolites of the fungus *Aspergillus flavus* that induce acute liver toxicity and liver cancer. Aflatoxins are bisfuranocoumarins that modify DNA forming AFB-*N7*-Guanine **[24]** and AFB-FAPy **[25]** adducts (Fig. 4) (Asao et al. 1963). Aflatoxins contaminate wheat, walnut, corn, cotton, peanuts, and tree nuts, affecting 25% of crops (Alshannaq and Yu 2017; Bullerman 1986; Jelinek et al. 1989; Severns et al. 2003). Individuals with liver cancer are more likely to have detectable levels of aflatoxin metabolites and DNA adducts in the urine with **[24]** ranging from 0.20 to 0.09 pg/mg creatinine (Qian et al. 1994; Ross et al. 1992).

Aristolochic Acid DNA Adducts

Aristolochic acids are a family of structurally related nitrophenanthrene carboxylic acids produced by Aristolochiaceae flowering plants, in widespread use as traditional herbal medicines (Michl et al. 2017). Aristolochic acid exposure is associated with an increased risk of nephropathy and urothelial cancers (Ivic 1969; Vanherweghem et al. 1993). Aristolochic acids are converted by nitroreduction to aristolactams which are further metabolized by P450s (Mix et al. 1982). These metabolites covalently modify DNA forming 7-(deoxyadenosin-N^6-yl) aristolactam I (dA-AL-I, **[26]**) and 7-(deoxyguanosin-N^2-yl) (dG-AL-I, **[27]**) (Fig. 5) (Stiborova et al. 1999). In formalin-fixed tissues, **[26]** was detected at 4.5–6.3 adducts/10^8 bases using LC-MS/MS (Yun et al. 2013).

N-nitroso Compound (NOCs) DNA Adducts

NOCs are produced from the reaction of amides and amines with nitrosating agents such as NO_2^-. NOCs are believed to be responsible for increased cancer incidence in rubber factory workers following exposure to nitrosodimethylamine (NDMA), which is hepatotoxic and carcinogenic in rats (Magee and Barnes 1956). NO_2^- is found in high concentrations in vegetables, processed meats, and grains and is associated with esophageal and brain cancers (Grosse et al. 2006). NOCs are alkylating agents and induce many DNA adducts including O^6-carboxymethyl-2′-dG (O^6-CM-dG, **[28]**) and O^6-methyl-guanine (O^6mG, **[29]**) (Fig. 5). **[29]** was measured by LC-MS/MS in mice

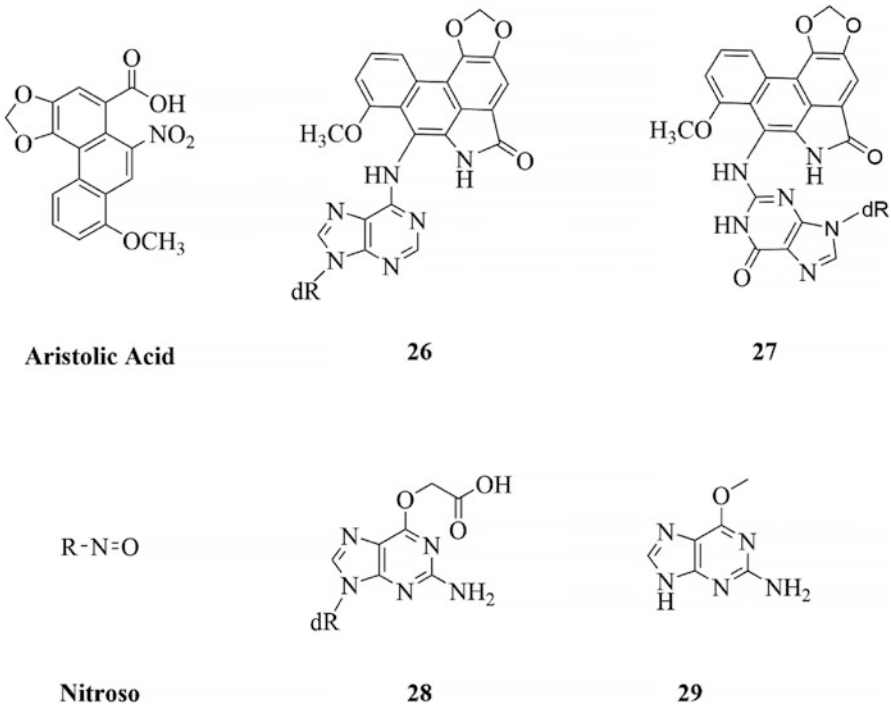

Fig. 5 Aristolochic acid and NOC adducts

following NOC exposure at levels of $300/10^7$ nucleotides in the liver and $50/10^7$ nucleotides in the colon (Kraus et al. 2019). These adducts have potential utility as biomarkers for NOC exposure and cancer risk.

Aromatic Hydrocarbon DNA Adducts

Benzene

Benzene is prevalent in the chemical industry as a solvent and is also a significant component of cigarette smoke (Wang et al. 2012). It is a hematopoietic toxin, increasing risk of aplastic anemia, myelodysplasia, and myelogenous leukemia (Aksoy 1989). Benzene is metabolized by cytochrome P450 CYP2E1 to benzene oxide and hydroquinone, which modifies DNA forming N^2-(4-hydroxyphenyl)-dG (N^2–4-HOPh-dG, **[30]**) (Fig. 6) (Bodell et al. 1996; Levay et al. 1991). Benzene oxide reacts directly with DNA forming 7-phenylguanine (7-PhG **[31]**) (Fig. 6) (Micova and Linhart 2012). **[31]** is detected with high sensitivity by LC-MS/MS (LOD, 8 amol, LOQ 40 amol) but does not correlate with smoking status (Zarth et al. 2014). Benzene-exposed workers displayed elevated etheno DNA adducts by LC-MS-MS, suggesting potential utility as biomarkers of benzene exposure (Li et al. 2015).

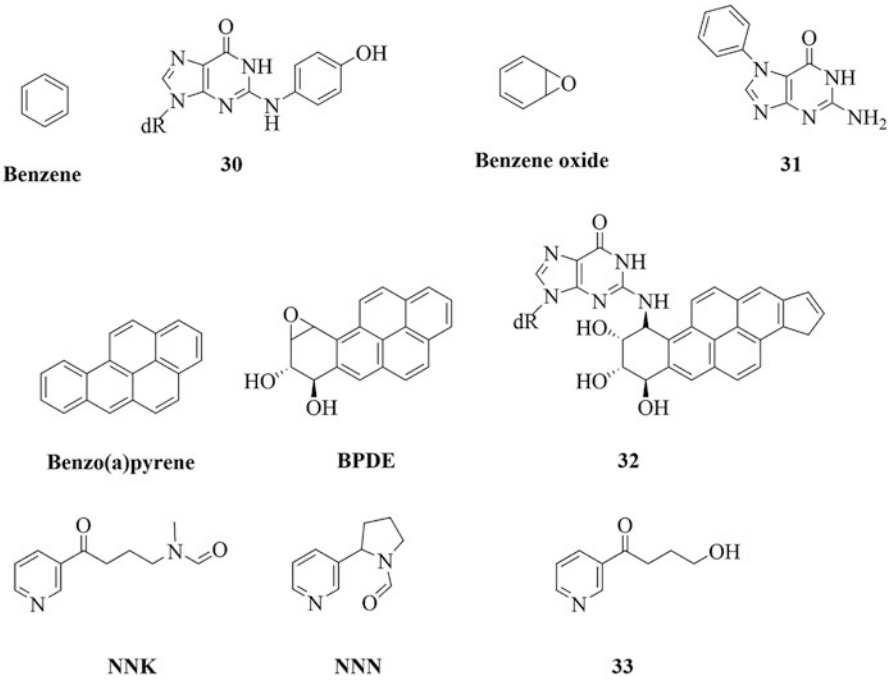

Fig. 6 Aromatic hydrocarbon adducts

Benzo[α]pyrene (BaP)

BaP is a carcinogen found in cigarette smoke and many environmental sources due to incomplete combustion (EPA 2017). BaP is metabolized by P450s CYP1B1, 1A1, and 2C19 to form reactive metabolites including trans-7,8-dihydroxy-anti-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) (Fig. 6) (Kim et al. 1998; Sulc et al. 2016). BPDE covalently modifies DNA, forming dG-BPDE **[32]** (Fig. 6) (Beland et al. 2005). Using a novel high-resolution LC-MS/MS method (LOD: 1 amol dG-BPDE), **[32]** was found to be 3x higher in smokers vs nonsmokers ($3.2/10^{11}$ vs $1.3/10^{11}$ nucleotides) (Villalta et al. 2017).

Nitrosamines

Nitrosamines include the strong carcinogens 4-(methylnitrosamino)-1-(3-pyridyl)-butanone (NNK) and *N*-nitrosonornicotine (NNN) (Fig. 6). NNK and NNN are metabolized by P450s CYP2A6 and 2A13 forming pyridyloxobutyldiazohydroxide, which covalently modifies DNA (Hecht 1999). Acid hydrolysis produces 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB, **[33]**), a biomarker for nitrosamine exposure (Fig. 6) (Hecht 1999). DNA isolated from mouthwash samples showed 12 pmol **[33]**/mg DNA in smokers and 0.23 pmol/mg in nonsmokers as measured by LC-MS/MS (Stepanov

et al. 2013). In buccal cells collected from cancer-free smokers and those with head and neck squamous cell carcinoma (HNSCC), **[33]** levels were 4.53 ± 14.36 pmol/mg DNA and 8.19 ± 18.12 pmol/mg DNA, respectively ($p = 0.002$) (Ma et al. 2016).

Carbonyl Adducts

Acrolein

Acrolein is an α,β-unsaturated carbonyl produced during combustion of wood, tobacco, foodstuffs, gasoline, and plastic and is also produced endogenously by lipid peroxidation and threonine, spermine, and spermidine catabolism (Fig. 7) (Esterbauer et al. 1991). Acrolein modifies dG at *N1* or N^2 forming cyclic propanodeoxyguanosine adducts, specifically 3*H*-6-hydroxy-3-(β-D-2′-deoxyribofuranosyl)-5,6,7,8-tetrahydropyrido[3,2-*a*]purin-9-one (6-OH-PdG or γ-OH-Acr-dGuo, **[34]**) and 3*H*-8-hydroxy-3-(β-D-2′-deoxyribofuranosyl)-5,6,7,8-tetrahydropyrido[3,2-*a*]purin-9-one (8-OH-PdG or α-OH-Acr-dGuo, **[35]**) (Fig. 7) (Chung et al. 1984). Using LC-MS/MS with an LOQ of 50 adducts/10^9 nucleotides, acrolein DNA adducts were significantly ($p = 0.025$) elevated in brain tissue isolated from patients with Alzheimer's disease (5150 ± 640/

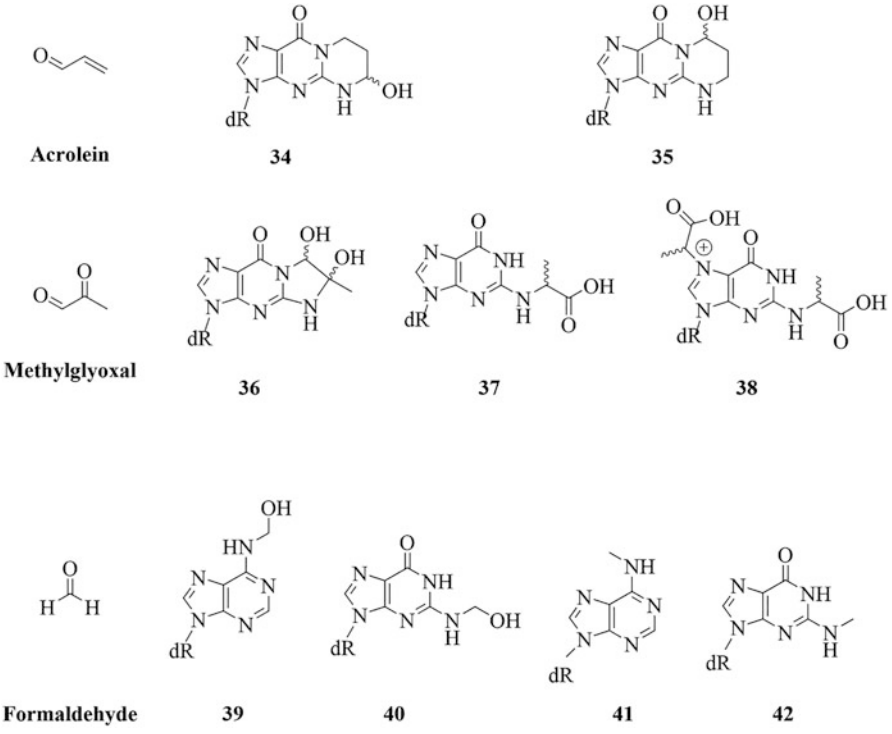

Fig. 7 Carbonyl adducts

10^9 nucleosides) compared to age-matched controls (2800 ± 460/10^9 nucleosides) (Liu et al. 2005). Comparison of adducts in smokers vs nonsmokers did not reveal significant differences (Zhang et al. 2007).

Methylglyoxal (MG)

Methylglyoxal is a reactive α-oxo-aldehyde produced from glycolysis, glycoxidation, threonine catabolism, and acetone metabolism (Fig. 7) (Thornalley et al. 1999). MG covalently modifies DNA to form the cyclic adduct 1,N^2-(1,2-dihydroxy-2-methyl)ethano-2′-dG (cMG-dG, **[36]**), N^2-carboxyethyl-dG (CEdG, **[37]**), and bis-adducted N^2-(1-carboxyethyl)-7–1-hydroxy-2-oxopropyl-dG (MG-CEdG, **[38]**) (Fig. 7) (Shuck et al. 2018). LC-MS/MS was used to quantify urinary **[37]** from individuals with and without T2D (Waris et al. 2015). In individuals with T2D, **[37]** was elevated twofold ($p < 0.001$) relative to nondiabetic controls (1.8 nmol/24 h vs 0.9 nmol/24 h) (Waris et al. 2015). In breast tumor tissue, **[37]** was significantly ($p = 0.003$) lower (6.6 ± 0.3/10^7 dG) compared to adjacent tissue (20.2 ± 1.6/10^7 dG) (Synold et al. 2008).

Formaldehyde

Formaldehyde is an industrial chemical that is also produced by tobacco, e-liquid, and gasoline combustion (Fig. 7) (Swenberg et al. 2013). Formaldehyde covalently modifies deoxyadenosine to form N^6-hydroxymethyl-dA (N^6-HOMe-dA, **[39]**) and deoxyguanosine to form N^2-hydroxymethyl-dG (N^2-HOMe-dG, **[40]**) (Fig. 7) (Lu et al. 2012; McGhee and von Hippel 1975; Zhong and Hee 2004). Reduction of these adducts with $NaCNBH_3$ during DNA hydrolysis leads to the more stable, measurable derivatives N^6-Me-dA **[41]** and N^{2-}Me-dG **[42]** (Fig. 7) (Wang et al. 2009). **[41]** measured in leukocyte DNA from smokers and nonsmokers revealed significantly higher ($p < 0.001$) adducts in smokers (179 ± 205 fmol/μmol dA) relative to nonsmokers (15.5 ± 33.8 fmol/μmol dA) (Wang et al. 2009). **[42]** was 19.5 ± 10.5/10^8 in nonsmokers and 26.0 ± 2 1.4/10^8 in smokers (ns).

Pesticides

Pesticides are widely used to improve agricultural crop yield. They vary in the specificity with which they target insects and other organisms (Angerer et al. 2007). There are several major categories of pesticides: organophosphates are esters of phosphoric acid and contain a phosphoryl or thiophosphoryl bond, carbamides are esters of carbamic acid, organochlorines are chlorinated hydrocarbon derivatives, and pyrethroids are man-made variants of the naturally occurring pyrethrins that are more selectively neurotoxic to insects than they are to humans (Angerer et al. 2007).

Pesticides of all classes, and particularly the organophosphate methyl esters, alkylate DNA at O^6 or *N7* of guanine and act as carcinogens. *N7*-methyl-2′-deoxyguanosine (*N7*-MedG, **[43]**) is a general biomarker of alkylating DNA damage (Fig. 8). O^6-methyl-2′-deoxyguanosine (O^6-MedG, **[44]**) forms at rates 10–100 times slower than *N7*-MedG but is significantly more carcinogenic and pro-mutagenic (Fig. 8) (Stocks et al. 2010; van Delft et al. 1997; Yang et al. 2002). **[43]** can be detected and quantified through ^{32}P postlabeling, ELISA, fluorescence, and immunological methods (Bianchini and Wild 1994). **[43]** in lymphocytes was significantly higher in Spanish greenhouse workers with high exposure to pesticides relative to those with low exposure ($p = 0.02$) (Gómez-Martín et al. 2015). A study of seasonal French field farmers used ^{32}P postlabeling to compare DNA adduct levels before (S0) and during (S4) heavy use of pesticides, with a non-farming referent group. In the referent group, no statistically significant difference was found between sampling periods in the mean relative adduct level (RAL): RALS0 = 3.5 ± 2.2x10^{-10}, RALS4 = 4.0 ± 3.7x10^{-10}, $p = 0.5$. (Significant increases in RAL were measured in the farmer group: RALS0 = 3.9 ± 3.4x10^{-10}, RALS4 = 13.3 ± 15.7x10^{-10}, $p = 0.0008$). Farmers

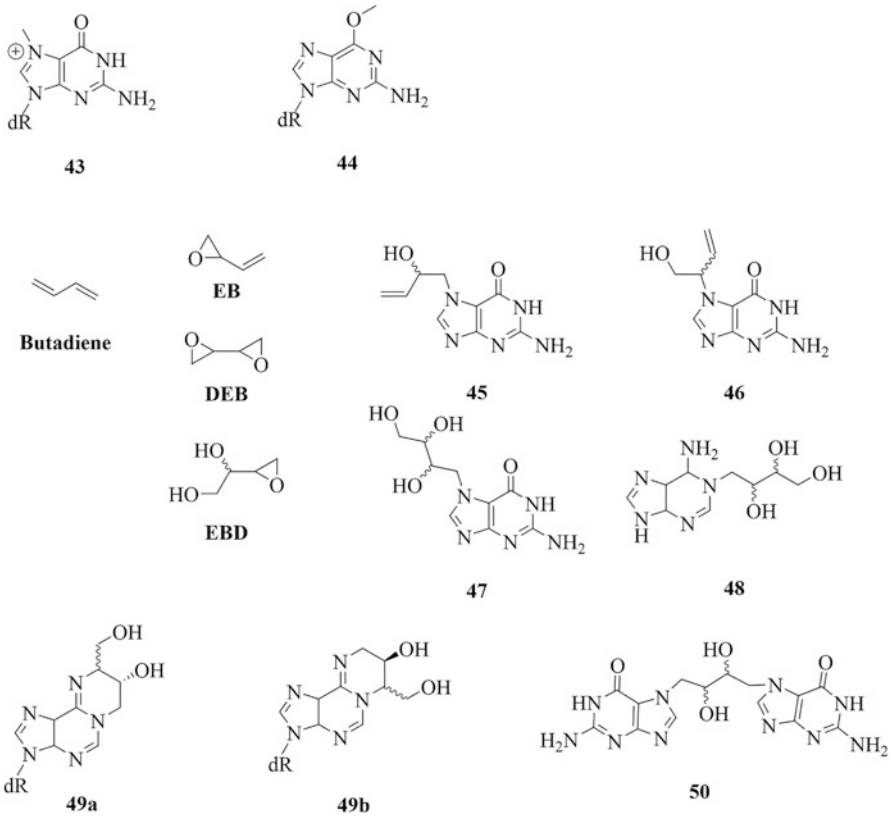

Fig. 8 Pesticides and butadiene adducts

had a significantly higher RAL relative to non-farmers only at S4 ($p = 0.02$) but not in S0. (Le Goff et al. 2005). Other studies have shown possible impacts of pesticide exposure on DNA adducts among farmers (Gallois et al. 2011; Peluso et al. 1996).

Butadiene

1–3-Butadiene (BD) is a flammable, toxic hydrocarbon produced from ethylene manufacturing and found in automobile exhaust and cigarette smoke (Fig. 8) (Brunnemann et al. 1989; Pelz et al. 1990; White 2007). BD is metabolized into three reactive electrophiles: 3,4-epoxy-1-butene (EB), 1,2,3,4-diepoxybutane (DEB), and 1,2-dihydroxy-3,4-epoxybutane (EBD) (Fig. 8). These electrophiles form covalent DNA adducts: *N7*-(2-hydroxy-3-buten-1-yl)-guanine (EB-GI, **[45]**), *N7*-(1-hydroxy-3-buten-2-yl)-guanine (EB-GII, **[46]**), *N7*-(2,3,4-trihydroxybut-1-yl)-guanine (*N7*-THBG, **[47]**), *N1*-(2,3,4-trihydroxybut-1-yl)-adenine (*N1*-THB-Ade, **[48]**), 1,N^6-(1-hydroxymethyl-2-hydroxypropan-1,3-diyl)-2′-deoxyadenosine (1,N^6-HMHP-dA, **[49]**), and 1,4-bis-(guan-7-yl)-2,3-butanediol (bis-*N7*G-BD, **[50]**) (Fig. 8) (Blair et al. 2000). **[47]** and **[45]** are the most abundant adducts. Cigarette smokers (8.20 ± 5.12 per 10^9 nucleotides) and nonsmokers (7.08 ± 5.29 per 10^9 nucleotides) did not have significantly different levels of **[47]** in human leukocyte DNA, suggesting that **[47]** is not associated with smoking (Sangaraju et al. 2013). However, **[47]** levels were significantly elevated ($p < 0.001$) in workers following occupational BD exposure (9.72 ± 3.80 per 10^9 nucleotides) relative to controls (3.08 ± 2.15 adducts per 10^9 nucleotides) (Sangaraju et al. 2013). Urinary **[46]** levels from nonsmokers (0.39 ± 0.13 pg/mg of creatinine) and smokers (0.59 ± 0.18 pg/mg of creatinine) were also not significantly different (Sangaraju et al. 2017), while urinary **[46]** from workers occupationally exposed to BD were significantly higher ($p < 0.01$, 1.25 ± 0.51 pg/mg of creatinine) compared to matched controls (0.22 ± 0.08 pg/mg of creatinine) (Sangaraju et al. 2017).

Chemotherapy-Induced DNA Adducts

Platinum (Pt)-Based Chemotherapy Drugs

Pt-based drugs are effective anticancer therapeutics for the treatment of testicular, ovarian, and colorectal cancers (Apps et al. 2015). The first Pt-based drug was cis-diamminedichloroplatinum(II) (cisplatin), approved by the FDA in 1979 (Fig. 9). Alternative Pt-based drugs, including oxaliplatin [1,2 diaminocyclohexane oxalatoplatinum(II)] and carboplatin [cis-diammine(1,1-cyclobutanedicarboxylato) platinum(II)], have also been approved (Fig. 9) (Apps et al. 2015). Platinum-based drugs form covalent bonds at the N^7 position of guanine, causing intra-strand cross-links that block replication and induce apoptosis. Cisplatin and carboplatin generate (NH_3)2Pt–d(pGpG) adducts (PtGG, **[51]**), while oxaliplatin forms [1,2-cyclohexanediamine]–Pt–d(pGpG) adducts (DACHPtGG, **[52]**) (Fig. 9). **[51]** in white blood

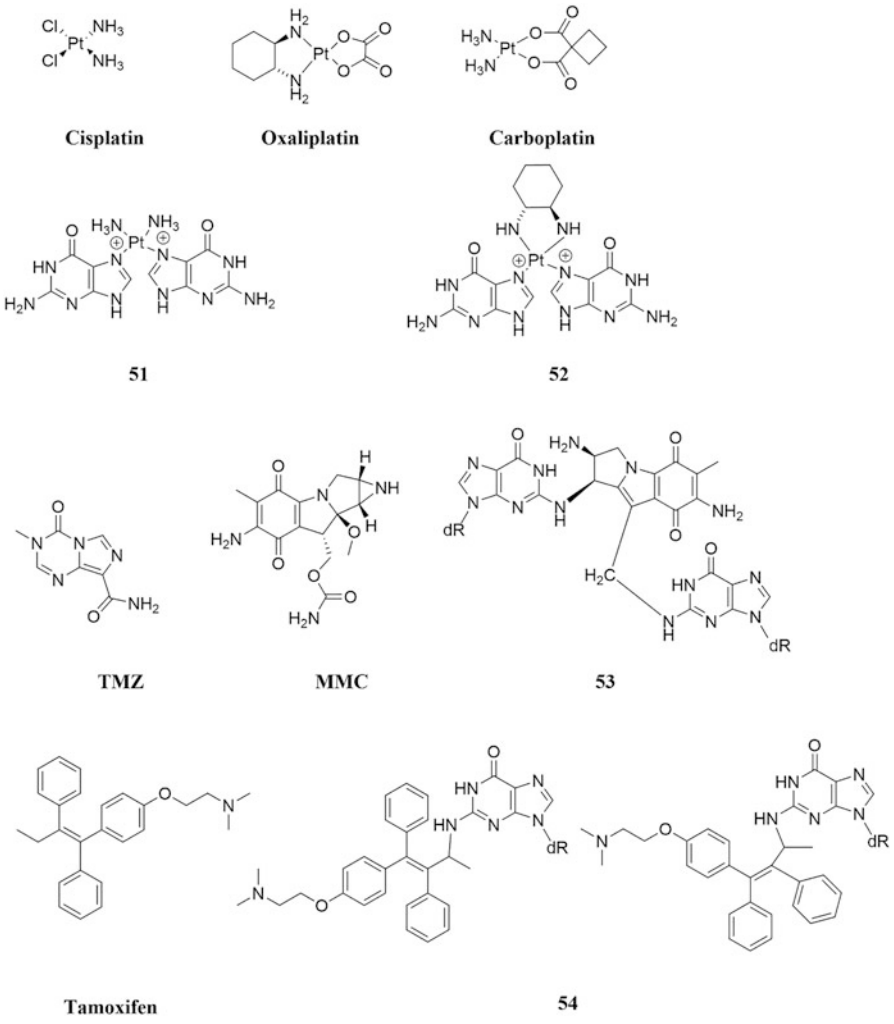

Fig. 9 Chemotherapy-induced adducts

cells isolated from patients with testicular cancer undergoing cisplatin-based chemotherapy increased from 0 adducts prior to treatment to 2.0 ± 1.3 fmol/ug DNA on day 5 of treatment, as measured using ELISA. **[51]** in tissue samples from the same study were 11.4 ± 0.2 fmol/ug DNA (Fichtinger-Schepman et al. 1990). Patients with non-small cell lung or bladder tumors given a microdose of ^{14}C-carboplatin (1.0×10^7 dpm/kg of body weight) before therapeutic doses of chemotherapy did not have significantly different **[51]** levels between responders and nonresponders. However, all patients with high average adduct levels (0.941 ± 0.030 monoadducts/10^8 nucleotides) responded to chemotherapy, while only 40% of those with low

average adduct levels (0.266 ± 0.158 monoadducts/10^8 nucleotides) did ($P < 0.0001$). Adduct levels were calculated by AMS (Zimmermann et al. 2017).

Temozolomide

Temozolomide (TMZ) is a DNA alkylating agent used to treat malignant gliomas, particularly glioblastoma multiforme and pituitary tumors (Fig. 9) (Syro et al. 2011). TMZ methylates purines at the O^6 and N^7 sites of guanine as well as the N^3 site of adenine. **[44]**, the most common lesion, is repaired by methylguanine methyltransferase (MGMT). Several methods have been devised to quantify TMZ-induced adducts, including immunofluorescence, immunoblot assays, and LC-MS/MS (Kraus et al. 2019). In HCT116 cells, **[44]** increased to 116 adducts/10^7 nucleotides after 4 h of exposure to 500 μM TMZ but decreased to 58 adducts/10^7 nucleotides 24 h posttreatment (Kraus et al. 2019). In a clinical trial, levels of **[44]** and **[43]** were measured by the standard MGMT activity assay procedure and immuno-slot blot, respectively, in peripheral blood mononuclear cells of patients with melanoma who had been given a TMZ dosage of 200 mg/m^2 five times a week for 4 weeks. Levels of **[44]** were found to be 3.9 fmol/ug DNA (95% CI: 1.3–7.1). Levels of **[43]** increased from 0.19 fmol/μg DNA (95% CI: 0–3.0) pre-dose to 55.9 fmol/μg DNA (95% CI: 34.1–79.3) during treatment (Kraus et al. 2019; Watson et al. 2009).

Mitomycin C

Mitomycin C (MMC) is an anticancer drug and cross-linking agent that forms covalent adducts at the N^2 position of guanine (Fig. 9) (Tomasz 1995). Adducts exist as MMC-G monoadducts and as inter-strand and intra-strand G-MMC-G cross-links **[53]** (Warren et al. 2001). MMC-DNA adducts were quantified using a ^{32}P postlabeling method in immortalized breast cancer cells xenografted onto mice and in stage IV breast cancer patient dermal metastases given a single dose of MMC at 5 or 10 mg/m^2. Maximum adduct levels of 2.59×10^{-6} total adducts/nucleotide and 0.349×10^{-6} cross-links/nucleotide for xenografted tissue and 3.46×10^{-7} total adducts/nucleotide and 0.76×10^{-7} cross-links/nucleotide for dermal metastases were observed (Warren et al. 2001). MMC-DNA adducts were quantified directly in human liver tissue through a ^{32}P postlabeling method and found to be $1–4 \times 10^{-8}$ adducts/nucleotide following 20 mg MMC (Kato et al. 1996).

Tamoxifen

Tamoxifen (TAM) is an estrogen analog used to treat estrogen receptor-positive breast cancer. TAM binds at N^2 in guanine, forming TAM-DNA adducts **[54]** (Fig. 9). Whether TAM is a genotoxic carcinogen in women is still under debate.

Rodent studies have established a correlation between tissue adduct levels and liver cancer, and TAM-DNA adducts have been measured in endometrium in patients with different conclusions regarding its carcinogenicity (Carmichael et al. 1996; Carthew et al. 2001; Shibutani et al. 2000). A recent study utilizing human endometrial and myometrial tissue from patients formerly diagnosed with breast cancer and treated with TAM measured levels of **[54]** through a chemiluminescence assay and found levels of $7.0 \pm 1.2/10^8$ nucleotides in normal tissue and 13.5 ± 1.4 adducts/10^8 nucleotides in malignant tissue, suggesting that TAM plays a mutagenic and genotoxic role in tumorigenesis (Hernandez-Ramon et al. 2014).

Applications to Prognosis

In this chapter, we review DNA damage induced by reactive molecules produced endogenously and exogenously and their potential utility as biomarkers of toxin exposure and disease risk. Several methods for measuring DNA adducts are reviewed, many of which have utility in clinical chemistry labs and for biomonitoring. These tests may be used to determine toxicological exposure, risk of disease onset and progression, and response to therapeutics. For example, measuring DNA damage induced by chemotherapeutic agents has implications for how well an individual may respond to treatment. Adduct levels determined for environmental toxins can be used to establish exposure limits for cancer risk.

Key Facts of DNA Biomarkers in Toxicology

Exposure to environmental agents produces reactive molecules that covalently modify DNA:

- These DNA modifications can be used as biomarkers to determine exposure.
- Multiple methods including mass spectrometry, ELISA, and ^{32}P postlabeling are used to measure DNA adducts.
- Chemotherapeutics induce DNA adducts, which can be used an indication of response to treatment.
- Biomarkers have utility in epidemiological studies to evaluate disease risk.

Mini-Dictionary of Terms

Biomarkers – molecules that can be used to measure exposure to specific agents and/or to determine the risk of disease

DNA adducts – covalent modifications of DNA induced by reactive molecules

Electrophiles – reactive molecules that can covalently modify macromolecules, forming stable adducts

Mass spectrometry – analytical method used to quantify adducts based on the mass/charge

Aldehydes – small volatile molecules containing a reactive carbonyl group

Summary Points

- DNA adducts formed by reactive molecules can be used to determine exposure to toxic compounds and the risk of developing disease.
- Several methods can be used to measure DNA adducts including mass spectrometry, immunohistochemistry, and P^{32} postlabeling.
- DNA adducts are associated with an increased risk of cancer.
- Urinary and serum DNA adducts positively correlate with exposure to toxic agents.
- Chemotherapeutic drugs induce DNA adducts, which can be measured to predict treatment efficacy.

Cross-References

► DNA Methylation as a Biomarker and Application to Aluminum: *ADRB2* 5′-Untranslated Region (5′-UTR) Methylation Level

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18

DNA Methylation as a Biomarker and Application to Aluminum: *ADRB2* 5′-Untranslated Region (5′-UTR) Methylation Level

Ola Elsayed Nafea

Contents

Abstract

Environmental heavy metal exposure might be considered as a contributor to childhood asthma development and its levels of control. Moreover, heavy metals have been linked to epigenetic changes, for example, DNA methylation in receptors incriminated in the pathology of asthma. One of these receptors is the β-2 adrenergic receptor (*ADRB2*). Aluminum exposure might cause a significant alteration in DNA methylation of the 5′-untranslated region (5′-UTR) of *ADRB2*. The *ADRB2* (5′-UTR) gene mirrors the responsiveness to β-agonists and childhood asthma level of control. The chapter focuses on the impact of aluminum on the *ADRB2* (5′-UTR) gene and the consequent effects on bronchial asthma.

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Keywords

5′-UTR · ADRB2 · *ADRB2* *5′*-untranslated region · Aluminum · Asthma control · β-2 adrenergic receptors · Bronchial asthma · DNA methylation · Epigenetics · Heavy metals · Untranslated regions · UTR region

Abbreviations

5′-UTR	*5′*-Untranslated regions
ADRB2	β-2 adrenergic receptor
GTP	Guanosine triphosphate
MBD2	Methyl-CpG-binding domain protein 2
MeCP2	Methylcytosine binding protein 2
UTRs	Untranslated regions

Introduction

Epigenetics is known as inherited variations in gene expression that are, distinctive from mutations, not related to changes in DNA sequences. The principal epigenetic mechanisms include DNA methylation, chromatin modification, loss of imprinting, and non-coding RNA (Handy et al. 2011; Sheikhpour et al. 2021). Epigenetic events are increasingly identified as fundamental mechanisms involved in cellular responses to environmental stressors and the underlying causes of diseases (Hansen et al. 2011; Baylin and Jones 2011). DNA methylation is the most frequently studied epigenetic mechanism and signifies a potential biomarker of future health consequences (Shanthikumar et al. 2020). DNA methylation, through 5-methyl- and 5-hydroxymethylcytosine (5mC and 5hmC), is one of the fundamental interfaces between the genome and environmental factors, and it can illustrate the phenotypic variations in human beings. It occurs almost at cytosine nucleotides in mammalian DNA. Common sites for DNA methylation involve promoter/enhancer areas of the affected gene. Chromatin-regulatory proteins (such as MeCP2 and MBD2) bind to the methylated cytosine nucleotides with less access of the transcriptional machinery to the gene, generating repressed chromatin domains (Jakopovic et al. 2013; Leenen et al. 2016; Vineis et al. 2017). Bronchial asthma is a complex illness that likely occurs as a result of interplay between various genetic and strong environmental influences (Duffy et al. 1990). Asthma has numerous negative health impacts in terms of mortality, morbidity, impairment of quality of life, and high healthcare costs (Leatherman 2015; Toskala and Kennedy 2015). Epigenetic changes can affect asthma development and control through different genetic pathways, albeit limited studies have been performed for addressing epigenetic events of asthma (Salam et al. 2012). Environmental pollution caused by exposure to heavy metals is increasingly becoming a major global health concern (Briffa et al. 2020). Modern human activities such as mining, manufacturing, and industrialization, in addition to synthetic materials used, led to environmental contamination by heavy metals.

Furthermore, it is too difficult to get rid of these metals from the environment because of their nonbiodegradable nature (Gautam et al. 2016). Excessive exposure to aluminum can lead to multisystemic adverse health effects including, but not limited to, neurological, hematological, and pulmonary adverse effects (Agency for Toxic Substances and Disease Registry (ATSDR) 2008).

Bronchial Asthma

Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role: in particular, mast cells, eosinophils, neutrophils (especially in sudden onset, fatal exacerbations, occupational asthma, and patients who smoke), T lymphocytes, macrophages, and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of coughing (particularly at night or early in the morning), wheezing, breathlessness, and chest tightness. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment (National Asthma Education and Prevention Program 2007). Bronchial asthma is a main non-communicable disease (NCD) affecting children as well as adults. Asthma affected about 300 million people in 2019 and was responsible for 461,000 deaths (Vos et al. 2020). By 2025, the number of asthmatic patients is predicted to be 400 million (Park et al. 2015). Heritability of asthma ranges from 25% to 80% according to the genetic studies of twins and asthmatic person families (Duffy et al. 1990). Numerous triggers are now well known to exaggerate asthma episodes such as infections, dust, air pollution, exposure to environmental toxins, microbes, allergens, weather changes, and animal fur and feathers (von Mutius 2009; Toskala and Kennedy 2015; Alizadeh et al. 2017).

The β-2 Adrenergic Receptor (ADRB2)

The human gene encoding *ADRB2* is located on chromosome 5q31 (Kobilka et al. 1987), a location genetically related to asthma and its associated phenotypes (Meyers et al. 2005; Postma et al. 2005). *ADRB2* is the gene encoding the receptor that has been largely investigated as a candidate gene for asthma susceptibility, asthma phenotypes, and response to therapy (Litonjua 2006; Hawkins et al. 2008) and the most reviewed pharmacogenetic loci among the β-agonist pathways. *ADRB2* gene has various polymorphic variants that were revealed in multiethnic genetic asthma patients (Hawkins et al. 2006; Ortega et al. 2014). *ADRB2* protein is a cell membrane-spanning receptor that binds adrenaline, but not adrenaline, dissimilar to the other adrenergic receptors, and therefore causes both bronchodilation and smooth muscle relaxation (Cherezov et al. 2007; Rosenbaum et al. 2007), whereas the role of *ADRB2* as an asthma susceptibility gene is still questionable (Raby and Weiss 2001; Thakkinstian et al. 2005; Liang et al. 2014). Similar to all G-protein-coupled receptors, the β2-receptor has seven transmembrane-spanning α-helices

(Liggett 2002). The β2-receptor is expressed in numerous cell types all over the body and is a key player in the control of cardiopulmonary, vascular, and neurological functions as well as the primary target of β2-agonist bronchodilators (Maciejewska-Skrendo et al. 2019; Abosamak and Shahin 2021). The receptor consists of eight alpha loops; three are extracellular, and five are intracellular. The receptor is N-glycosylated at amino acids 6, 15, and 187; these are vital for incorporation into the cell membrane and for agonist-elicited receptor trafficking (Mialet-Perez et al. 2004). At amino acid 341, the cysteine of the human β2-receptor is palmitoylated to anchor the carboxy-terminus to the membrane (O'Dowd et al. 1989). The area between the seventh transmembrane-spanning domain and the palmitoylated cysteine is also an α-helix, sometimes considered as the fourth intracellular loop (Johnson 2006). β2-receptors are present in two forms, activated and inactivated, and equilibrium between both forms exists, with the inactivated form being prevalent (Liggett 2002). Activation of β2-receptor occurs in association with the α-subunit of the Gs protein, along with guanosine triphosphate (GTP) molecule, whereas inactivation of β2-receptor occurs through the replacement of the GTP by guanosine diphosphate with a subsequent reduction in the α-subunit affinity for the receptor. β2-receptors agonists probably have their effects not through stimulation of receptor conformational alteration but rather by attachment as well as temporarily stabilizing receptors in their activated form (Onaran et al. 1993). In vitro exposure to glucocorticoids is associated with an increase in the number of β2-receptors, while their numbers are declined following exposure to an agonist, approximately for more than 2 h. In addition, following virus infection or exposure to pro-inflammatory cytokines, for example, interleukin 1-β (Collins et al. 1988; Koto et al. 1996; Shore et al. 1997; Taylor and Hancox 2000).

Aluminum

Aluminum is the third most abundant metal in the earth's crust and is an extensively consumed metal in the world (Kaizer et al. 2008; Mohseni et al. 2016). Exposure to aluminum is nearly inevitable secondary to its wide spread in the environment (Kumar and Gill 2009). Aluminum is widely utilized in many aspects of our daily life. Aluminum-containing food additives are considered as a main source of aluminum in the diet (Soni et al. 2001; Saiyed and Yokel 2005). Other sources of aluminum exposure include, but not limited to, vaccine adjuvants, antacids, cosmetics, cooking utensils, and drinking water because of water treatment processes. Aluminum coming out from weathering rocks and soils or rocks and soils caused by pollution-induced acid rain is considered a natural source of aluminum. Moreover, aluminum may be a contaminant in several foodstuffs such as infant formulae, dairy products, and drinks such as juice, wine, and tea. In industrial fields, aluminum is enormously utilized because of its excellent physical and chemical characters. Therefore, the general population is at risk for aluminum exposure through inhalation of ambient air and ingestion of contaminated foodstuff and water as well as through dermal exposure (Lin et al. 1997; Niu 2018; Bichu et al. 2019).

Aluminum and *ADRB2* 5′-Untranslated Region (5′-UTR) Methylation Level

In eukaryotes, mature mRNA has two untranslated regions: a 5′-untranslated region (5′-UTR) and a 3′-untranslated region (3′-UTR); each is a coding region composed of triplet codons that each encodes an amino acid Mignone et al. 2002) (Fig. 1). The 5′-untranslated region (5′-UTR) is a regulatory region of DNA located at the 5′ end of all protein-encoding genes that is transcribed into mRNA not protein. 5′-UTRs have diverse regulatory elements; additionally, 5′-UTRs play a key role in the control of translation initiation (Bradnam and Korf 2008). DNA methylation at CpG sites close to promoter regions is strongly related to gene expression in both the stably expressed and developmentally controlled genes, albeit this relays on CpG intensity (Martino and Saffery 2015). ADRB2 is the primary target of both short- and long-acting β-2 adrenergic receptor agonists in asthma therapies. *ADRB2* 5′-UTR methylation modification mirrors the responsiveness to β-agonists and childhood asthma level of control. Increased blood *ADRB2* 5′-UTR methylation is associated with an increased risk for severe childhood asthma (Fu et al. 2012). DNA methylation provides a possible mechanism for environmental modification of genetic responses, e.g., aluminum exposure, as those at the *ADRB2* locus. A 407-base pair inconsistently methylated CpG island overlaps the *ADRB2* 5′-untranslated region (5′-UTR) and may represent a crucial source of epigenetic regulation of this gene (Gaffin et al. 2014; Nafea et al. 2020). Aluminum can perform epigenetic and imprinting functions, such as clamping DNA and chromatin proteins (Lukiw et al. 1998; Bryant et al. 2004). Aluminum is reported to have a genotoxic effect and can cause DNA changes and epigenetic modifications (Darbre 2005). Aluminum exposure in the occupational setting caused a significant alteration in DNA methylation with a subsequent increase in serum aluminum levels (Yang et al. 2015). Aluminum binds to the sugar-phosphate backbone of the DNA under neutral pH and causes DNA changes (Zhang et al. 2002). *ADRB2* gene alteration has been linked to the severity and level of control of bronchial asthma; however, some studies have revealed conflicting findings. In addition, epigenetic change in the (*ADRB2*) could play a prominent role in asthma phenotype (Gaffin et al. 2014). DNA methylation is the most studied epigenetic mechanism of childhood asthma. The epigenetic basis of childhood asthma included specific methylation signatures linked to airways and immune cells allergic inflammatory reactions, revealing a regulatory role for methylation in asthma pathogenesis (Gomez 2019). In the same context, aluminum induces the secretion of inflammatory cytokines by human alveolar macrophages in vitro (Braydich-Stolle et al. 2010). A study by Guo et al. (2013) showed a significant positive association between inflammatory cytokines and plasma

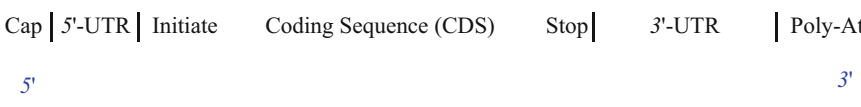

Fig. 1 A schematic representation of the untranslated regions in mature mRNA in humans

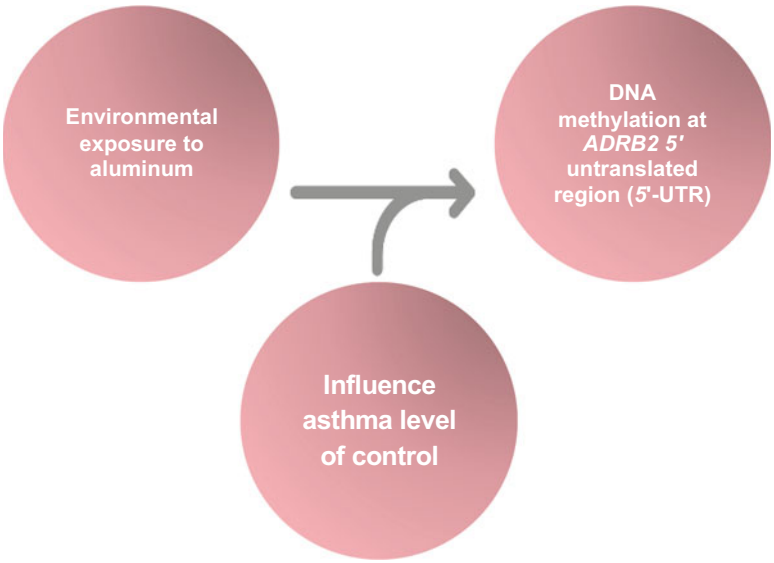

Fig. 2 Environmental exposure to aluminum can affect β-2 adrenergic receptor gene (*ADRB2*) at a 5′-untranslated region (5′-UTR) with a subsequent change in asthma control level

aluminum levels in asthmatic patients. Nafea et al. (2020) proposed a causal relationship between the increased blood aluminum concentration and *ADRB2* 5′-UTR hypermethylation (Fig. 2).

Bronchial Asthma and Epigenetic Events

Environmental heavy metal exposure might be considered as a contributor to childhood asthma development and its levels of control. It has been linked to epigenetic changes, for example, DNA methylation in receptors implicated in the pathology of asthma. One of these receptors is the β-2 adrenergic receptor (*ADRB2*) (Guo et al. 2013; Ray et al. 2014; Wu et al. 2019; Nafea et al. 2020). Alteration of *ADRB2* 5′-UTR methylation reflects the response to β-2 adrenergic receptor agonist treatment and the control of bronchial asthma in children. For example, DNA hypermethylation is directly related to asthma severity and level of control (Fu et al. 2012). Conversely, DNA methylation in the *ADRB2* gene is linked to decreased asthma severity (Gaffin et al. 2014). Table 1 summarized studies concerned with DNA methylation at *ADRB2 5′-UTR* in childhood asthma. Epigenetic alterations can regulate clinical manifestations of childhood and adulthood asthma; in addition, a synergic effect between environmental asthma precipitating factors and epigenetic events coexists (Sheikhpour et al. 2021). Environmental influence-gene interaction has the potential to modify disease risks during vulnerable developmental periods, e.g., childhood, with the resultant long-lasting epigenome

Table 1 Summary of studies concerned with DNA methylation at *ADRB2 5′-UTR* in childhood asthma

References	Environmental factor	Epigenetic event	Phenotype
Fu et al. (2012)	Nitric oxide exposure	DNA hypermethylation	Increased severity of asthma
Gaffin et al. (2014)	–	DNA methylation	Decreased severity of asthma
Nafea et al. (2020)	Aluminum exposure	DNA hypermethylation	Increased severity of asthma

changes with subsequent altered pulmonary and/or immune system functions. Children are more vulnerable to toxic exposure than adults since they have comparably more food contaminant intake, active development, and several exposure pathways and are more prone to socio-behavioral influences such as hand-to-mouth behavior making them at risk of ingestion of a diversity of contaminants (Au 2002). Most human studies have examined the relations between epigenetic events, mainly DNA methylation, with respiratory disorders (Fu et al. 2012; Gaffin et al. 2014; Krauss-Etschmann et al. 2015). Epigenetic modification in association with asthma has a vital role in addressing the response to drug therapy. Currently, different epigenetic mechanisms are being explored as biomarkers for asthma control and novel therapeutic intervention (Comer et al. 2015; Nafea et al. 2020).

Potential Applications to Prognosis

In this chapter, the level of asthma control is influenced by exposure to aluminum and *ADRB2 5′*-UTR methylation level (Nafea et al. 2020). Recognition of environmental and non-environmental sources of aluminum exposure is crucial to address preventive strategies and plans to reduce aluminum-related negative health outcomes, especially for children. *ADRB2 5′*-UTR methylation level could be used a potential prognostic biomarker for severity and risk stratification in asthmatic patients.

Mini-dictionary of Terms

- **Epigenetics** – heritable phenotypic variations without changes in DNA sequence.
- **DNA methylation** – a biological process in which a methyl group is inserted to the 5′ position of the pyrimidine ring of cytosine nucleotides located close to a guanine nucleotide, which is known as CpG sites, to form 5-methylcytosine (5mC).
- **Untranslated regions (UTRs)** – are loci on the mRNA before the initiation codon (five primer untranslated region, 5′-UTR, or leader sequence) and after

the stop codon (three primer untranslated region, *3′*-UTR, or trailer sequence) that are not translated.
- **Genome –** the total genetic materials of an organism.
- **Bronchial asthma** – a chronic inflammatory disorder of the airways characterized by bronchial hyper-reactivity and a different degree of respiratory obstruction.

Key Facts

- DNA methylation is one of the fundamental interfaces between the genome and environmental factors affecting the phenotypic variations in human beings.
- Environmental pollution caused by exposure to heavy metals is increasingly becoming a major global health concern.
- Environmental heavy metal exposure might be considered as a contributor to childhood asthma development and its levels of control.
- Excessive exposure to aluminum can lead to multisystemic adverse health effects.
- *ADRB2* gene has been largely investigated as a candidate gene for asthma susceptibility, asthma phenotypes, and response to therapy

Summary Points

- Epigenetics is known as inherited variations in gene expression that are, distinctive from mutations, not related to changes in DNA sequences.
- DNA methylation is the most frequently studied epigenetic mechanism and signifies a potential biomarker of future health consequences.
- Bronchial asthma is a complex illness that likely occurs as a result from interplay between various genetic and strong environmental influences.
- Exposure to aluminum is nearly inevitable secondary to its wide spread in the environment.
- Increased blood aluminum concentration might alter DNA methylation at *ADRB2 5′*-UTR.
- Epigenetic modification in association to asthma has a vital role in addressing the response to drug therapy.

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Autophagy as a Biomarker of Cytotoxicity

19

Seishiro Hirano

Contents

Abstract

Since autophagy is involved in both cytoprotective and cytostatic/cytotoxic pathways depending on the types of stressor and culture conditions, autophagic biomarkers represent both favorable and unfavorable cellular responses to intracellular and extracellular signals. Biomolecules in the autophagy machinery are also used in the other cellular processes, which may misdirect autophagy in its pro-death/pro-survival responses of cells under chemical or physical stresses. After reviewing the current understanding of the mechanisms of autophagy and the role of autophagy molecules, pros and cons of using these molecules as biomarkers of cytotoxicity are discussed in this chapter.

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Keywords

Autophagy · Xenophagy · Cytotoxicity · Pro-survival · Ferroptosis · LC3-II · p62/SQSTM1 · Lysosome · Autophagosome · Autolysosome · Autophagolysosome · Amphisome · Reactive oxygen species

Abbreviations

3-MA	3-Methyladenine
AMPK	5′ Adenosine monophosphate-activated protein kinase
CMA	Chaperon-mediated autophagy
DMA^{5+}	Dimethylarsinic acid
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
KEAP1	Kelch-like ECH-associated protein 1
LAP	LC3-associated phagocytosis
LC3	Light chain 3
MMA^{3+}	Monomethylarsonous acid
mTORC1	Mammalian target of rapamycin complex I
Nrf2	Nuclear factor erythroid 2-related factor 2
PE	Phosphatidylethanolamine
PtdIns(3)P	Phosphatidylinositol 3-phosphate
RFP	Red fluorescent protein
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SQSTM1	Sequestosome 1
Ub	Ubiquitin
VPS34	Vacuolar protein sorting 34

Introduction

Since autophagy literally means “self-eating,” the cellular response to autophagy and its consequences are diverse. Unfavorable cytosolic components such as denatured macromolecules and damaged organelles are removed by autophagy, and some molecules are recycled and reused. Recently, it has been reported that aberrant stress granules, cytosolic non-membrane organelles, are also degraded by autophagy (Franzmann and Alberti 2021). Under starvation, cells can regenerate nutrients by autophagy processes. In this context, autophagy is cytoprotective, and dysfunction of autophagy leads to the pathogenesis of many diseases (Doherty and Baehrecke 2018). In contrast to this pro-survival function, excessive autophagy and unregulated autophagy result in the loss of cellular components and cause cell death. This chapter focusses on the latter type of autophagy, because the pro-death nature of autophagy is not well understood, and changes of autophagic biomarkers in the pro-death condition are still controversial.

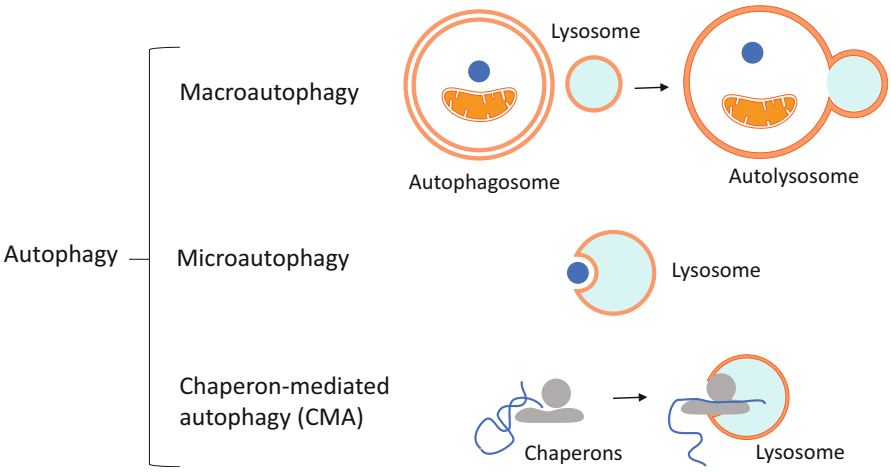

Fig. 1 Three types of autophagy. Cytosolic cargos are finally digested by lysosomes in the autophagy pathway. Macroautophagy features the fusion of a double-membrane autophagosome with a lysosome. Microautophagy is initiated by invagination of the lysosome membrane. Chaperons assist transportation of denatured substances from the cytosol to lysosomes in chaperon-mediated autophagy

There are three types of autophagy: macroautophagy, microautophagy, and chaperon-mediated autophagy (Parzych and Klionsky 2014; Schuck 2020). These autophagy types are functionally similar to each other, as cytosolic cargos are eventually digested by lysosomes (Fig. 1). However, the modes of uptake by lytic organelles such as lysosomes and late endosomes are different among these types. Hereafter, classical macroautophagy is simply called autophagy, because unlike the other two types, macroautophagy features the generation of double-membrane autophagosomes, and biomolecules involved in autophagosome membrane biogenesis are closely related to cytotoxicity.

Autophagy and Its Membrane Biogenesis

Autophagy flux proceeds through the following three steps, and many autophagy-related molecules are involved in each step (Fig. 2):

1. Initiation/nucleation (phagophore)
2. Elongation/maturation/closure (autophagosome)
3. Fusion with lysosome (autolysosome)

LC3-II, a lipidated form of the microtubule-associated protein light chain 3 (LC3-I), and p62 (also known as sequestosome 1, SQSTM1) are two autophagy-related biomolecules that play an important role in the biogenesis of autophagy membranes (Pankiv et al. 2007). p62 binds ubiquitylated proteins via the

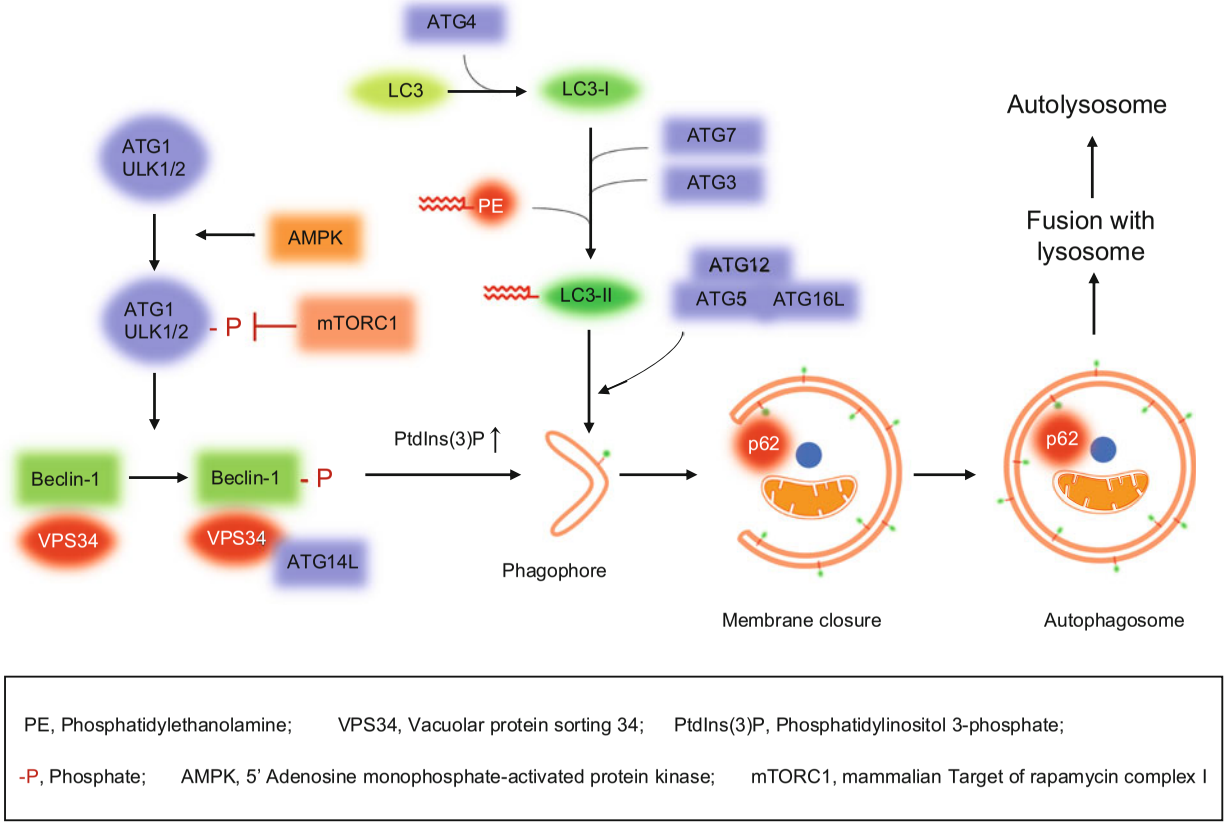

Fig. 2 (continued)

ubiquitin-associated domains and LC3 through its LC3-interacting region (Tyedmers et al. 2010). LC3-II on the outer (cytosolic) side of autolysosome is recycled, while LC3-II on the inner (luminal) side is degraded (Bauckman et al. 2015). Autophagy flux, which is maintained at a low level in normal conditions, is activated in response to various cellular stress such as starvation (Shibutani et al. 2015), radiation (Bristol et al. 2012), and exposure to environmental toxicants and pharmaceutical drugs in eukaryotes (Tables 1 and 2).

Selective Autophagy, LC3-Associated Phagocytosis (LAP), and Amphisomes

As autophagy means "self-eating," autophagy cargos are supposed to be intracellular materials. When the cargo is specified, selective names are assigned to autophagy such as mitophagy (mitochondria) and pexophagy (peroxisomes). Xenophagy is a selective autophagy process in which foreign materials such as particles and microorganisms are ultimately digested in autophagolysosomes (Bauckman et al. 2015; Heckmann and Green 2019). Even though the cargos of xenophagy are extracellular substances like pathogens, xenophagy is a type of autophagy because the phagocytosed bacteria or pathogens that are free in the cytosol are taken up by autophagosomes (Fig. 3).

One should be careful using LC3 as a biomarker of autophagic cell death with LAP. It is well recognized that phagocytosed foreign substances are ultimately digested in phagosomes, but LAP is a new type of phagocytosis and a non-canonical form of autophagy in which some autophagy machinery components are used (Fig. 3). In this context, it is hard to differentiate LAP from xenophagy, except that phagosomes have a single membrane while autophagosomes have a double membrane. p62 seems not to be involved in LAP. The fusion product of LAP and lysosomes is termed the autophagolysosome. In addition, phagosomes can be sequestered by autophagosomes. The fusion product of this phagosome-in-autophagosome and lysosome is also called an autophagolysosome (Klionsky et al. 2014). However, the involvement of macropinocytosis in xenophagy remains to be elucidated (Hirano and Kanno 2015).

Occasionally, an autophagosome fuses with an endosome, generating what is called an amphisome, before autophagosome cargos are digested in autolysosomes. Amphisome formation is another autophagic pathway in which extracellular foreign materials are digested by autolysosomes (Fig. 4).

Fig. 2 Autophagy membrane biogenesis and autophagy flux. The initial step of autophagy is the formation of a phagophore, which probably originates from the ER-Golgi. The phagophore grows and bends, while lipidated LC3 molecules (LC3-II) attach to phagophore membranes. Damaged organelles and aggregated proteins are engulfed by this double-membrane structure. Finally, the membrane is closed generating an autophagosome, which is fused with a lysosome, and the cargos are digested by lytic enzymes in the autophagosome. The whole process is called autophagy flux. Many molecules are involved in this process. ULK1/2 is a mammalian homologue of yeast ATG1

Table 1 Changes in LC3 and p62 levels following exposure to environmental toxicants

Chemicals	Cell type	Viability ↓	LC3-II or LC3-II/LC3-I ↑	LC3 puncta ↑	p62 ↑ or ↓	Reference
Gd^{3+}	HEK293 (human kidney cell)	100 μM	50–100 μM		Not changed up to 800 μM	Takanezawa et al. (2020)
Cu^{2+}	GC-1 (germ cell)	10 μM	100 μM		50 μM ↑	Kang et al. (2019)
CuO nanoparticle	HUVEC (human endothelial cell)	5 μg/mL	20 μg/mL	20 μg/mL (12 h)	20 μg/mL ↑	Zhang et al. (2018)
CuO nanoparticle	A549 (lung carcinoma)	0.3 μg/mL (24 h)	30 μg/mL	30 μg/mL		Sun et al. (2012)
Cd^{2+}	AML-12 (mouse hepatocyte)	5 μM (12 h)	5 μM (12 h)	5 μM (12 h)	5 μM (12 h) ↑	Zhou et al. (2019)
Cd^{2+}	Immortalized rat renal cell		5 μM (1–5 h)	5 μM (5 h)		Chargui et al. (2011)
Cd^{2+}	Rat mesangial cell	2 μM (24 h)	2 μM (24 h)		2 μM (24 h) ↓ (FBS was depleted in 48 h preculture)	Fujishiro et al. (2018)
As^{3+}	MIN6 (pancreatic cell)	1–4 μM	4 μM		4 μM ↓	Wei et al. (2020)
As^{3+}	Rat cortex neuron	5 μM (24 h)	3 μM 24 h 5 μM 8–48 h	5 μM 16 h		Teng et al. (2015)
As^{3+}	Priess (human B lymphoblastoid cell)		1.5 μM (8 days)			Bolt et al. (2012)
Mn^{2+}	PC12 (rat pheochromocytoma cell)	100 μM (24 h)	300 μM (12 h)		300 μM (12 h) ↑	Zhou et al. (2018)
ZnO nanoparticle	A549 (human lung carcinoma cell)	30 μg/mL (24 h)	30 μg/mL		30 μg/mL ↑	Zhang et al. (2018)

ZnO nanoparticles	Mouse peritoneal exudate macrophage	2.5 μg/mL (24 h) apoptosis	2.5 μg/mL (12 h)	2.5 μg/mL (12 h)		Roy et al. (2014)
ZnO nanoparticle Zn^{2+}	J774.1 (mouse macrophage)	ZnO 5 μg/mL (24 h) Zn^{2+} 90 μM (24 h)	ZnO 5 μg/mL (18 h) Zn^{2+} 180 μM (18 h)	Not clear	ZnO 5 μg/mL (18 h) ↑	Hirano and Kanno (2020)
TiO_2 (18 nm) particles	HaCaT (human keratinocyte)	25 μg/mL (24 h)	25 μg/mL (24 h)	25 μg/mL (1 and 24 h)		Lopes et al. (2016)
Nano-silica	L-02 (human hepatocyte) HepG2 (human hepatoma)	25 μg/mL (12–24 h) 25 μg/mL (24 h)	12.5 μg/mL (24 h) 25 μg/mL (24 h)	12.5 μg/mL (24 h) 12.5 μg/mL (24 h)	Not changed up to 100 μg/mL (24 h) 25 μg/mL (24 h) ↑	Wang et al. (2017)
Quantum dots (CdSe, InGaP)	LLC-PK1 (porcine kidney)	CdSe 10 nM (24–48 h) InGaP 100 nM (24–48 h)	CdSe 10 nM (6 h) InGaP 100 nM (6 h)			Stern et al. (2008)
Ti_3C_2 Mxene quantum dots	HUVEC (human endothelial cell)	50 μg/mL (24 h)	25 μg/mL (24 h)		25 μg/mL (24 h) ↑	Gu et al. (2021)
SPIO (supermagnetic iron oxide) nanoparticle	L-02 (human hepatocyte)	60 μg/mL (12 h)	15 μg/mL (12 h)		15 μg/mL (12 h) ↑	He et al. (2019)
Amorphous silica	HUVEC (human endothelial cell)	25 μg/mL (24 h)	50 μg/mL (24 h)	50 μg/mL (24 h)	50 μg/mL (24 h) ↑	Guo et al. (2016)
CH_3Hg^+	SH-SY5Y (human neuroblastoma) Rat cerebral cortical neuron	5 μM (24 h) 1 μM (12 h)	5 μM (24 h) (SH-SY5Y)	10 μM (4 and 24 h) (SH-SY5Y)	2 μM (12 h) ↑ (rat neuron)	Lin et al. (2019)

(continued)

Table 1 (continued)

Chemicals	Cell type	Viability ↓	LC3-II or LC3-II/LC3-I ↑	LC3 puncta ↑	p62 ↑ or ↓	Reference
Lindane (organochlorine pesticide)	Primary rat hepatocyte	50 μM (48 h) 25 μM (72 h)	25–75 μM (24–72 h)	50 μM(48 h)		Zucchini-Pascal et al. (2009)
Polybrominated diphenyl ethers (flame retardants)	HepG2 (human hepatoma)		25 μM (24–48 h)	0.1 μM (24 h)	5–25 μM (24–48 h) ↑	Pereira et al. (2017)
2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)	MDBK (Madin-Darby bovine kidney cell)	1 pg/mL (8–24 h)	0.01 pg/mL (12 h) 1 pg/mL (8 h)			Fiorito et al. (2011)

Table 2 Changes in LC3 and p62 levels following exposure to pharmaceutical drugs and bioactive substances

Chemicals	Cell type	Viability ↓	LC3-II or LC3-II/LC3-I ↑	LC3 puncta	p62 ↑or ↓	Reference
Lipopolysaccharide (LPS)	RAW (mouse macrophage)		200 ng/mL (12 h)	200 ng/mL (12 h)	200 ng/mL (12 h) ↑	Yin and Cao (2015)
Lipopolysaccharide (LPS)	J774.1 (mouse macrophage)		1 μg/mL (18 h)		1 μg/mL (18 h) ↑	Hirano and Kanno (2020)
Erastin (ferroptosis inducer)	Fibroblast (human, mouse)	2–5 μM (24 h)	2–5 μM (24 h)		Not changed clearly	Park and Chung (2019)
MG132 (proteasome inhibitor)	HGPS-fibroblast	10 μM (24 h)	5 μM (36–48 h)			Harhouri et al. (2017)
Bortezomib (proteasome inhibitor)	Cal-78, SW-1353 (human chondrosarcoma cell)	2.5 nM Cal-78 (48 h) 5 nM SW-1353 (48 h)	2.36 nM for Cal-78 (24 h) 4.95 nM for SW-1353 (24 h)	2.36 nM for Cal-78 (24 h) 4.95 nM for SW-1353 (24 h)		Lohberger et al. (2016)
Tertiary amines	U937 (human monocyte)	1–1000 μM (48 h)	1–1000 μM (6 h)			Parks and Marceau (2016)
H_2O_2	ARPE-19 (retinal pigment epithelial)	125 μM (24 h)	500 μM (8 h)		500 μM (8 h) ↑ (both protein and mRNA levels)	Sheu et al. (2019)
Amphotericin B	GRX (hepatic stellate cell)	1.25 μg/mL (24 h)		1.25 μg/mL (24 h)		Uribe et al. (2013)

(continued)

Table 2 (continued)

Chemicals	Cell type	Viability ↓	LC3-II or LC3-II/LC3-I ↑	LC3 puncta	p62 ↑or ↓	Reference
Hypoxia MPP^+	SH-SY5Y (human neuroblastoma)	0.1% oxygen 4 h MPP+ (0.6 mM, 24 h)	1% oxygen hypoxia 8 h MPP^+ (2 mM, 24 h)	1% hypoxia (8 h) 1–2 mM MMP^+ (24 h)		Tzeng et al. (2010)
Docosahexaenoic acid (DHA, ω-3 fatty acid)	Colorectal cancer cell lines	70 μM (48 h)	70 μM (48 h)		70 μM (24 h) ↑	Samdal et al. (2018)
Ethambutol (antituberculosis drug)	RGC-5 (rat retinal ganglion cell)	3 mM (24 h)	4 mM (24 h)	4 mM (24 h)	4 mM (24 h) ↑	Huang et al. (2015)
Crotoxin (rattle snake venom)	SK-MES-1 (human lung squamous carcinoma)	25 μg/mL (24–48 h)	50 μg/mL (24–48 h)		50 μg/mL (24 h) ↓	Han et al. (2014)
Desmethylclomipramine (DCMI, metabolite of anti-depressant clomipramine)	HeLa (human cervical cancer cell)		10 μM (2 h)	1–10 μM (0.5–4 h)	5–10 μM (24 h) ↑	Rossi et al. (2009)

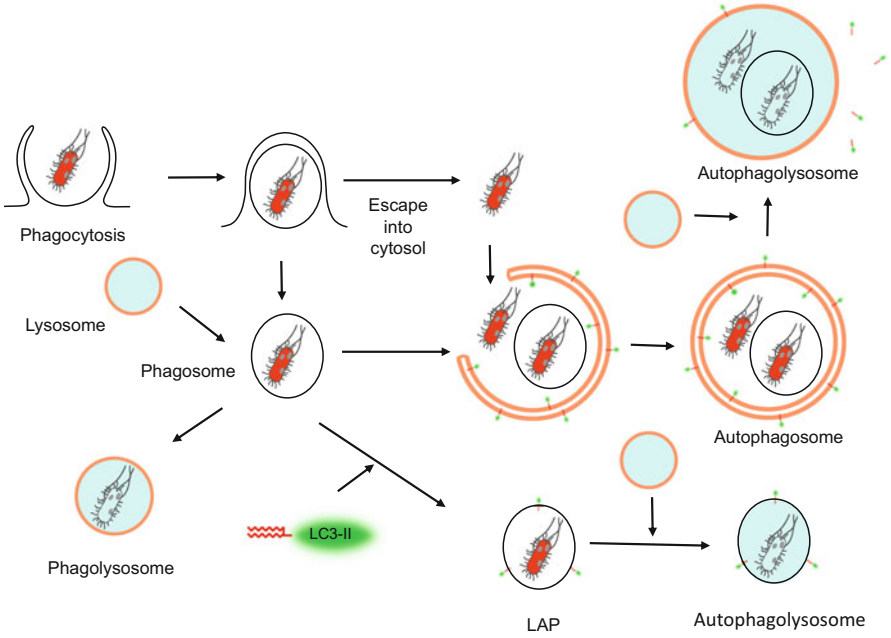

Fig. 3 Large extracellular substances like pathogens are phagocytosed and digested by phagolysosomes or autophagolysosomes. Phagocytosis is initiated by the protrusion of plasma membrane around a foreign substance. After closure of the membrane protrusion, a single-membrane phagosome is internalized with its cargo. The phagosome is fused with a lysosome, and the cargo is digested by lytic enzymes in the phagolysosome. In some cases, lipidated LC3 molecules are attached to the phagosomal membrane before fusion with a lysosome. The product is called an LC3-associated phagosome (LAP). The fusion product of LAP and a lysosome is called an autophagolysosome. When the phagosome and escaped pathogens are trapped by an autophagosome and the autophagosome is fused with a lysosome, the product is also called an autophagolysosome. The autophagolysosome is the final stage of the xenophagy flux

Measurement of Autophagy Flux

The conversion of LC3 from LC3-I to LC3-II has been commonly used to measure autophagic activity. The conversion is assayed by immunoblot analysis; LC3-I migrates at 18 kDa, and LC3-II migrates at 16 kDa in regular SDS-PAGE, although the molecular weight of LC3-II is higher than that of LC3-I by lipidation. Since LC3-II is more stable than LC3-I, the LC3-II level may be more reliable than the LC3-II/LC3-I ratio in immunoblot analysis depending on the preparation and storage condition of the lysate samples.

Microscopic detection of LC3 dots have been used together with the LC3-I to LC3-II conversion to measure autophagy activity after immunostaining cells with anti-LC3 antibody or by ectopically expressing fluorescence-tagged LC3 in the cells. However, LC3 protein tends to aggregate in an autophagy-independent manner

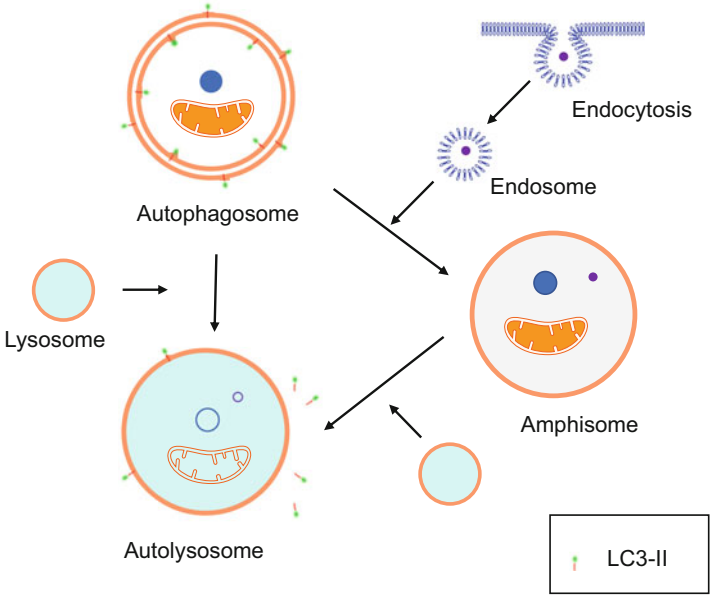

Fig. 4 Autophagosomes and amphisomes. Cells can internalize foreign small substances (< 200 nm) by endocytosis. Endocytosis is initiated by the invagination of plasma membrane, and an endosome is generated when the invaginated cavity is pinched off. Autophagosome can fuse with an endosome before fusion with a lysosome, and the product is called an amphisome. An autolysosome that originates from an amphisome contains endosomal cargo. Since endosomal cargos are foreign substances, autophagy via amphisomes is in part xenophagy. LC3-II molecules of the inner autophagosome membrane are digested by lytic enzymes, while those of the outer membrane are recycled

when the tagged protein is transiently overexpressed, and therefore LC3 dots do not always represent autophagic structures (Kuma et al. 2007). RFP/mCherry-GFP-LC3 tandem constructs have been widely used to differentiate autophagosomes from autolysosomes. The principle is based on RFP fluorescence being relatively stable in acidic conditions (autolysosomes), while GFP fluorescence is easily quenched in autolysosomes, and thus autophagosomes are labeled yellow, and autolysosomes are labeled red in the construct-transduced cells. However, the counting of autophagosomes alone does not necessarily reflect autophagy flux, because both the induction of autophagy and the dysfunction of lysosomes increase the number of autophagosomes. Several methods to monitor autophagy cascades have been developed, and some of them are applicable in vivo (Mizushima and Murphy 2020).

The p62 protein level can be used as a biomarker of autophagy activity. However, p62 is not stable in living cells, with a half-life in normally growing HeLa cells estimated to be 6 h (Bjorkoy et al. 2009). Moreover, p62, a Ub-chain binding protein, is an immediate early response gene product, and both transcription and protein levels are increased by stimulants such as growth factors and phorbol ester (Lee et al. 1998).

Since p62 is a substrate for digestion by autophagy, the p62 level appears to be increased when the lysosomal acidic pH is neutralized by alkalinization drugs (Schuck 2020).

Cyto-ID, a cationic amphiphilic tracer dye, has been used recently. Autophagic compartments can be labeled in a high-throughput manner with minimal staining of lysosomes by this dye (Guo et al. 2015). Monodansylcadaverine has also been used with LysoTracker® to monitor autolysosome-dependent fluorescence (Akhtar et al. 2020). However, non-specific fluorescence images can be obtained when cells are exposed to insoluble particles, because particles accumulate in lysosomes and their surfaces have the potential to scatter light (Hirano and Kanno 2020). Thus, fluorescence monitoring with monodansylcadaverine and LysoTracker® alone is not recommended for late autophagy monitoring when insoluble particles accumulate in the lysosomes.

Cytotoxic and Cytoprotective Roles of Autophagy

Autophagy is a catabolic process whereby misfolded protein aggregates and damaged organelles are eliminated, and therefore autophagy serves a survival or protective cellular role. Mitophagy, a selective autophagic process of mitochondria, should be cytoprotective, because this type of autophagy removes damaged mitochondria to maintain cell homeostasis (He and Klionsky 2009). L-asparaginase-induced autophagy is a good pro-survival example. The loss of asparagine, which is essential for cell growth and protein glycosylation for cell adhesion, reduces cell invasion and increases LC3-II without cytotoxicity in ovarian cancer cells and endothelial cells, suggesting that autophagy may forestall anoikis, a form of apoptosis caused by the loss of adhesion (Yu et al. 2012). Docosahexaenoic acid (DHA), an ω-3 fatty acid, is known as an anticancer agent and cytotoxic at 70 μM. Less DHA-sensitive LS411N cells have a higher level of basal autophagy and LC3-II levels than DHA-sensitive DLD-1 cells, indicating that the high basal autophagy is pro-survival (Samdal et al. 2018). Platinum nanoparticles (< 1 nm) cause DNA damage in trophoblasts. But the cytotoxic and genotoxic effects are reduced by autophagy flux, at least in part by decreasing the amount of platinum nanoparticles reaching the nuclei, suggesting that autophagy plays a protective role in platinum nanoparticle-exposed cells (Nakashima et al. 2019).

However, cells undergo autophagy gene-related death (type-II programmed cell death) if the autophagy level exceeds a physiological range (Chatterjee et al. 2014; Pattingre et al. 2005). The autophagy inhibitors chloroquine (20 μM) or ConA (10 nM) reduce oxidative stress (H_2O_2)-induced cell death, indicating that H_2O_2 causes autophagic cell death (Sheu et al. 2019). In MCF-7 breast tumor cells, ionizing radiation promotes autophagy that is cytoprotective, because pharmacological or genetic interference of autophagy results in growth suppression and apoptosis. However, a low concentration of chloroquine (5 μM), an autophagy flux inhibitor, also protects cells from the combination of vitamin D ($1,25D_3$) and

radiation (Bristol et al. 2012). Thus, autophagy plays dual roles in irradiated breast cancer cells.

Favorable and undesirable outcomes after the induction of autophagy may signify two sides of the same coin, and whether autophagy is beneficial or not for the survival of toxicant-exposed cells is still an enigma. How can the activation of autophagy lead to pro-death or type-II programmed cell death? Ferritinophagy, a selective form of autophagy, is one plausible mechanism for autophagy-related cell damage (Parzych and Klionsky 2014). When ferritin is degraded in autolysosomes, the intracellular iron level is increased, and reactive oxygen species (ROS) are generated via Fenton's reaction (Fig. 5). Increased intracellular ROS levels ultimately result in serious cell damage (Park and Chung 2019). It has been reported that As^{3+} induces autophagy (ferritinophagy), increases mitochondrial ROS, and causes pancreatic dysfunction through ferroptosis (iron-mediated apoptosis), suggesting that ferroptosis is a form of autophagic cell death in pancreatic cells (Wei et al. 2020). In the following section, the relationship between cytotoxic signals and autophagy biomarkers is discussed.

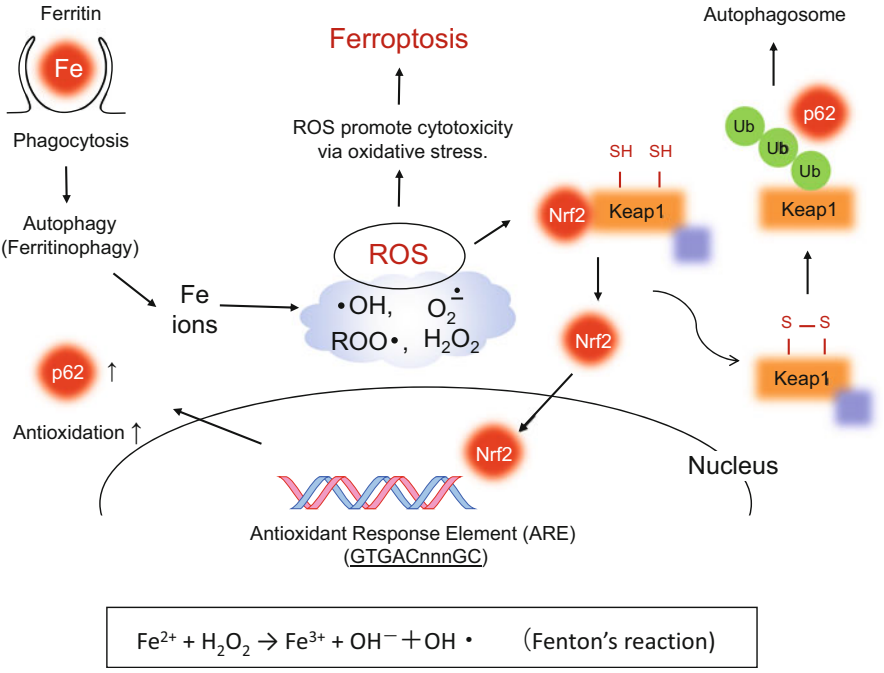

Fig. 5 Oxidative stress and autophagy-mediated cell death. Ferritinophagy is a type of xenophagy in which extracellular ferritin is processed by the autophagy machinery. Since ferritinophagy increases intracellular iron ions, ROS such as hydroxy radicals are generated via Fenton's reaction and induce cell death, which is called ferroptosis. Reciprocally, ROS activate the Nrf2 transcription factor and increase p62 together with antioxidant enzyme levels to enhance autophagy

Biomarkers in Autophagy-Induced Cytotoxicity

Several lines of evidence suggest that p62 and LC-3-II levels in immunoblotting and LC3 puncta formation in immunostaining or fluorescent microscopic analyses are hallmarks of autophagy progression. Increases in the LC3-II/LC3-I ratio and the formation of LC3 puncta and changes in p62 are commonly seen in a variety of cell types when the cells are exposed to cytotoxic substances. Cytotoxic levels of environmental toxicants and pharmaceutical drugs and changes of the autophagic biomarkers in cells exposed to these chemicals are summarized in Tables 1 and 2. Autophagy plays a pro-death role in arsenic-exposed primary cultured rat cortical neurons. MMA^{3+}, the most toxic arsenical, more effectively increases LC3-II than As^{3+}, a moderately toxic arsenical. As^{5+} and DMA^{5+}, less toxic arsenicals, do not change either cell viability or LC3-II levels at 5 μM (Teng et al. 2015).

One should keep in mind, however, that these autophagy markers are also increased by lysosome-autolysosome inhibition (Wang et al. 2017). As such, autophagosomal markers reflect either the increase of autophagosome formation or the impairment of degradation of autophagosomes. In principle, p62 decreases when autophagy activity is increased, and vice versa, because p62 is a substrate of autophagic degradation (Han et al. 2014).

LC3 and p62 are most intensively studied in toxicology, mostly because the amounts of these proteins and the lipidation of LC3 change sharply in response to acute toxicity. Some studies indicate that these autophagic markers respond to toxicants before cell membrane damage or cell death. However, these changes may be consequences of lethal cell damage rather than indicators for early injury of cells. The LC3-II level and the number of LC3 puncta increase only when cells are exposed to lethal levels of environmental toxicants as shown in Table 1.

Autophagy-induced pro-death signaling may depend on the cell types and exposure conditions. Autophagy impairment with 3-MA and wortmannin alleviates CuO nanoparticle-induced death in A549 cells (Sun et al. 2012). In contrast, however, autophagy impairment with 3-MA and wortmannin and knockdown of beclin-1 do not alleviate cell death caused by CuO nanoparticles in HUVEC cells (Zhang et al. 2018).

Conclusions and Implications for Autophagy Biomarkers of Cytotoxicity

The conversion of LC3-I to LC3-II is considered to be a benchmark process in the stabilization and growth of autophagic membranes. However, the LC3-I to LC3-II conversion appears to occur when cells are seriously damaged by any type of chemical. There is crosstalk between autophagy and other modes of cell death (Gump and Thorburn 2011; Orrenius et al. 2013). As such, increases in LC3-II and p62 levels and the LC3-II/LC3-I ratio can be consequences of crosstalk between autophagy and cell death. It is important to differentiate the formation of autophagosomes and the activation of autophagy/autophagy flux, especially when alkalinization chemicals are used and lysosomal function is impaired.

Applications for Prognosis of Other Diseases or Conditions

Since autophagy is an intracellular event, it may be hard to use "autophagic biomarkers of cytotoxicity" for prognosis. However, monitoring the turnover rates of autophagy-related biomolecules can be a useful tool for the prognosis of neurodegenerative diseases and cancer, because cancer cells use autophagy machinery to survive, and the dysfunction of autophagy results in neurodegenerative diseases such as amyotrophic lateral sclerosis, Alzheimer's disease, and Parkinson's disease.

Mini-dictionary of Terms

- **Autophagy flux**. The sequential process from the generation of autophagosome membranes and the digestion of autophagic cargo by autolysosomes.
- **Mode of cell death**. There are several modes of cell death: necrosis, apoptosis, necroptosis, pyroptosis, and ferroptosis. Autophagic cell death is close to apoptosis, because intracellular components are retained in the membrane structures. Autophagic cell death is often called "type II apoptosis," while canonical apoptosis is "type I."
- **Internalization of extracellular components**. Cells can internalize large solid particles by phagocytosis and liquids and small particles by macropinocytosis or endocytosis. The plasma membrane extrudes by polymerization of actin fibers in the case of phagocytosis and macropinocytosis, while endocytosis is initiated by invagination of the plasma membrane to internalize extracellular substances.
- **LC3 puncta**. The activation of autophagy can be visualized by immunostaining of LC3 or forced expression GFP-LC3. Since LC3-II is associated with autophagosome membranes, LC3 is observed as puncta or dots by microscopy.
- **LC3-II/LC3-I ratio**. In response to autophagy stimulation, LC3-I is lipidated with phosphatidylethanolamine to generate LC3-II, which easily associates with membranes. Thus, the LC3-II/LC3-I ratio is often used as a marker of autophagy membrane biogenesis and the activation of autophagy.

Key Facts of Autophagy Membrane Biogenesis

The first step of autophagic membrane biogenesis is the formation of a phagophore.

The source of the phagophore membrane is ER-Golgi.

The phagophore membrane curves as it grows, making a double-membrane curvature.

Impaired biomolecules and organelles are surrounded by the membrane curvature.

Two ends of the membrane curvature fuse to generate an autophagosome.

Summary Points

- Autophagy is a physiological intracellular degradation process.
- Autophagy is dysregulated in various human diseases.
- Autophagy influx is a biomarker of the development of diseases.
- An increased level of LC3-II is a good biomarker of cytotoxicity.
- p62 is a hallmark of autophagy, but may not be a good biomarker of cytotoxicity.

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Erythrocyte Glutathione Transferase P1-1 as a Biomarker in Environmental Toxicology: A New Narrative

20

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Contents

Abstract

The erythrocyte glutathione transferase (e-GST) is the most abundant GST isoenzyme in the red blood cells. This enzyme is able to inactivate numerous

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different toxic compounds, and many studies indicate its possible role as a biomarker in environmental toxicology since it is hyper-expressed in case of exposure to pollutants in humans as well as in animals. This chapter summarizes most of these studies, revealing also a peculiarity of this biomarker: many toxins induce hyper-expression of e-GST with consequent increased activity, but a few toxins are actually inhibitors of e-GST and cause a decrease in activity. Studies involving inhabitants, mammalians reared in polluted areas, miners, or other workers exposed to a variety of toxic chemicals, demonstrate that a simple, rapid, and inexpensive analysis of e-GST activity may provide an early alarm signal of contamination that can be verified with more traditional chemical analyses to characterize the nature of the contaminants.

Keywords

Biomarker · Glutathione transferase · Erythrocyte glutathione transferase · Blood toxicity · Environmental toxicology · Pollutant · Animal model · Detoxifying activity · Antioxidants · Xenobiotics · Enzyme inhibition

Abbreviations

CDNB	1-Chloro-2,4-dinitrobenzene
CKD	Chronic kidney disease
DNDGIC	Dinitrosyl-diglutathionyl-iron complex
e-GST	Erythrocyte glutathione transferase
GSH	Glutathione
GSTP1-1	Glutathione transferase class P1-1
GSTPi	Glutathione transferase class P isoforms
GSTs	Glutathione transferases
PMs	Particulate matters
RBCs	Red blood cells

Introduction

The discovery of glutathione S-transferases (GSTs; E.C. 2.5.1.18), an enzyme that is active in the metabolism of carcinogens and other toxic compounds dates back to Booth et al. (1961). The GSTs comprise a complex enzyme superfamily that has been subdivided further into a number of classes based on a variety of criteria, including amino acid sequence, and immunological, kinetics, and tertiary-quaternary structural properties. Today, the mammalian cytosolic GSTs are grouped into seven classes: alpha, zeta, theta, mu, pi, sigma, and omega (Oakley 2011). Interclass sequence identities are around 25% or less. Despite the low sequence identities, all cytosolic GSTs share a common fold, which is also largely conserved in mitochondrial GSTs. Mammalian cytosolic GSTs are dimeric enzymes; usually, the dimers are made from identical chains, but heterodimers made of two different chains from the same class are also known.

GSTs are able to inactivate a wide range of xenobiotics and endogenous metabolic by-products, thus promoting their conjugation to glutathione (GSH). The GSH-toxin conjugation makes many toxins less reactive and more water-soluble, which facilitates their excretion via the phase III detoxification pathway (Salinas and Wong 1999). A few isoenzymes also display a glutathione-dependent peroxidase activity and catalyze isomerization reactions (Hayes et al. 2005).

Interestingly, human GSTP1-1 has been shown to catalyze the isomerization of 13-*cis*-retinoic acid to all-*trans*-retinoic acid (Chen and Juchau 1998). Since this isomerization is independent of GSH, it is distinct from most other isomerization reactions reported for GSTs, which usually require GSH.

A few typical reactions catalyzed by GSTs are shown in Fig. 1.

Nonenzymatic activity is also involved as GSTs may act like "ligandins" binding toxins and promoting their excretion (Sheehan et al. 2001). Mammalian GSTs, in particular the GSTP1-1, have intracellular roles as modulators of Jun N-terminal kinase signalling pathways, protecting cells against hydrogen peroxide–induced apoptosis (Tew and Ronai 1999).

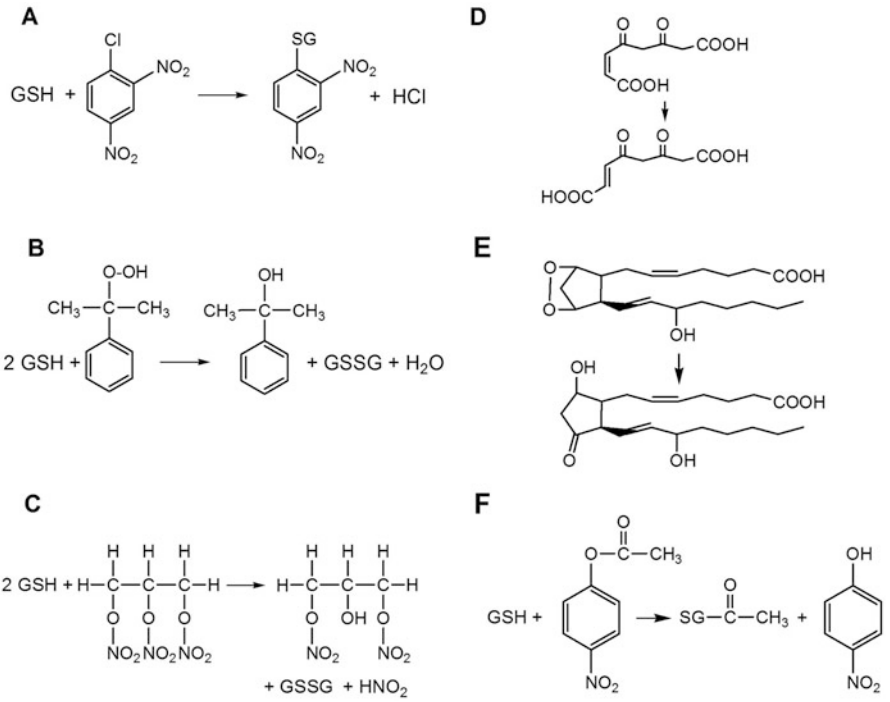

Fig. 1 Some examples of reactions catalyzed by GSTs. (**a**) Conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB). (**b**) Glutathione peroxidase activity toward cumene hydroperoxide. (**c**) Reduction of trinitroglycerin. (**d**) Isomerization of maleylacetoacetate. (**e**) Isomerization of prostaglandin H_2 (PGH_2) to prostaglandin D_2 (PGD_2). (**f**) Thiolysis of 4-nitrophenylacetate. (Figure modified from Hayes et al. (2005))

During the last decades, a number of reviews have collected and organized the immense number of scientific studies on GSTs, and a few of them are mentioned in the reference section of this chapter (Allocati et al. 2018; Armstrong 1997; Dirr et al. 1994; Eaton and Bammler 1999; Edwards et al. 2000; Hayes and Pulford 1995; Hayes et al. 2005; Salinas and Wong 1999; Sheehan et al. 2001; Wilce and Parker 1994).

GSTs and Toxin Exposure

GST enzymes are known to respond to exposures to toxins. Many studies reported activity alterations of GSTs in various organs of different living organisms after exposure to industrial metals or in soils contaminated with pesticides (Aly and Schröder 2008; Bocedi et al. 2019; Enayati et al. 2005; Gonçalves et al. 2021; Hellou et al. 2012; Saint-Denis et al. 2001). Recent studies highlighted GSTs as responders to xenobiotics from many chemical classes, including both inorganic and organic compounds, and in particular agrochemicals (Bundy et al. 2008; Stott et al. 2001). This review chapter focuses more on the erythrocyte GSTP1-1 (e-GST) and in particular on its emerging role as a biomarker in environmental toxicology.

The Peculiar Molecular Properties of GSTP1-1

GSTP1-1 is a dimeric protein composed of two identical subunits with a total of about 23,000 Da (Fig. 2). Each subunit contains a binding site for GSH, named the G-site, and a second site (the H-site) able to bind many different electrophilic toxic organic compounds. A third site, at the dimer interface, is involved in the binding

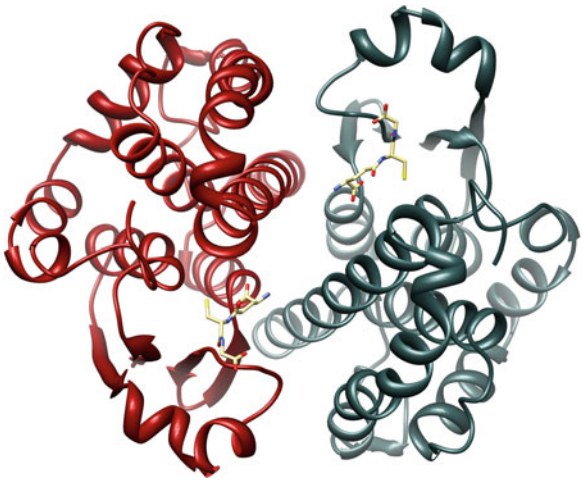

Fig. 2 Three-dimensional structure of GSTP1-1. GSTP1-1 (erythrocyte glutathione transferase) (PDB id: 6gss). The two monomers are in dark red and dark slate grey ribbons. Glutathione molecule is reported in ball-and-stick according to atom type

and transport of toxins out of the cell. The rate-limiting step of the most common catalyzed reaction, i.e., the conjugation of GSH to dangerous organic compounds, is represented by the nucleophilic attack of the sulfur atom of GSH to the electrophilic center, promoted by a lowered pK_a of the sulfhydryl group of GSH, which is shifted from 9.1 to 6.5. This unusually low pK_a is caused by the proximity of a Tyr residue, which forms a hydrogen bond with the sulfhydryl group of GSH. X-ray crystallographic data and molecular dynamic simulations demonstrate that a high flexibility of the alpha helix 2, flanking the G-site, is crucial for the conjugation of GSH to the co-substrate (Fig. 2). A singular molecular feature of GSTP1-1 is the presence of four cysteine residues, which remain in the reduced form in the native enzyme. The role of all these residues is not yet clear, but Cys47 displays unusual properties. It is characterized by a very low pK_a (3.5), which makes it possible for the thiolate ion to form a strong ion pair with the protonated ε-amino group of Lys54 at physiological pH values (Lo Bello et al. 1993). This link seems to be essential for the correct structure of helix 2. Chemical modification of this cysteine as well as its oxidation to form a disulfide with Cys101 causes a drastic loss of enzyme activity (Fabrini et al. 2014; Sluis-Cremer et al. 1996).

Figure 1 shows the reaction between GSH and 1-chloro-2,4-dinitrobenzene (CDNB), the most common co-substrate used for kinetic studies on GSTP1-1. The kinetic pathway follows a random sequential mechanism. This reaction, which can be followed spectrophotometrically at 340 nm where the product absorbs ($\varepsilon = 9600\ M^{-1}\ cm^{-1}$), is universally used to measure GST activity in biological samples (Habig and Jakoby 1981). Alternatively, a fluorometric procedure has been also developed using 4-chloro-7-nitrobenzofurazan as co-substrate (Ricci et al. 1994). Among the GST superfamily, GSTP1-1 is one of the most studied isoenzymes. During the last 40 years, crystallographic, NMR, fluorescence, and kinetics studies have been applied to reveal the peculiar molecular properties of this homodimer enzyme. Of particular interest is the tight binding of the two subunits ($K_d < 10^{-10}$ M) and their cooperative interaction, which have important physiological consequences (Fabrini et al. 2009). Since 1989, the unusual nonequivalence of the two identical subunits in their reaction with sulfhydryl reagents has been studied and assigned to an inter-subunit communication effect. However, only in 1995 was it demonstrated that the mutation of Cys47 or Lys54 into alanine disclosed a true positive cooperativity in GSTP1-1 in its interaction with GSH. This cooperative modulation is latent in the native enzyme, but it becomes evident in the case of point mutations or under particular temperature-stress conditions (Caccuri et al. 1999). In fact, GSTP1-1 is also present in the upper layers of the epidermis, where it plays a protective role against carcinogens and toxic compounds that may get in contact with the skin or is exposed to low or high temperatures that can alter its catalytic efficiency (Caccuri et al. 1999). A sophisticated mechanism of temperature adaptation has been developed for this enzyme during evolution and finalized to maintain an efficient catalytic activity of GSTP1-1. In fact, above 35 °C, this enzyme displays positive cooperativity for GSH binding, whereas negative cooperativity occurs below 25 °C. This homotropic mechanism minimizes changes in GSTP1-1 affinity for GSH due to temperature fluctuation. This is likely an advantage for epithelial

skin cells, which are naturally exposed to temperature variations and, incidentally, also to carcinogenic compounds that always need efficient detoxifying systems (Caccuri et al. 1999). As a whole, GSTP1-1 represents the first enzyme, which displays such temperature-dependent homotropic regulation of its substrate (e.g., GSH) binding.

A second cooperative interaction was described for GSTP1-1 after observing the interaction of this enzyme with the dinitrosyl-diglutathionyl-iron complex (DNDGIC), a natural carrier and storage molecule of nitric oxide. This complex is formed when NO is overproduced, but it is also toxic for the cells. GSTP1-1 and other GST isoenzymes are able to protect the cell binding DNDGIC with extraordinary high affinity ($K_d = 10^{-9}$ M) and thus making it harmless. However, binding of this complex to GSTP1-1 causes a strong inhibition of this enzyme. A complete inactivation is limited by a negative cooperative mechanism; i.e., when a first subunit has bound the DNDGIC, the second adjacent subunit becomes much less affine. This self-preservation ability has been observed even in the presence of other toxic compounds, and it represents one of the most plausible examples of utility given by a negative cooperativity mechanism in enzymes (Bocedi et al. 2016a).

Activity Detection of GSTP1-1 in Biological Samples: Critical Points

The method most used to quantify the activity for GSTP1-1 is the one described by Habig and Jakoby (1981). This procedure uses CDNB as co-substrate and is based on the spectrophotometric absorbance at 340 nm of the conjugation product between GSH and CDNB, the *S*-(2,4-dinitrophenyl)glutathione ($\varepsilon = 9600\ M^{-1}\ cm^{-1}$), a reaction catalyzed by all GSTs (Fig. 1). In this assay, the GSH level is saturating (1 mM), while CDNB is under-saturating given that its concentration in the assay (1 mM) is similar to the K_m value. Thus, the enzyme rate cannot exceed 50% of the maximum velocity obtainable under saturating conditions. Unfortunately, higher CDNB concentrations cannot be used due to its scarce solubility in aqueous solutions. Thus, the Habig procedure needs special attention because even minor variations in the CDNB concentrations give significant differences in the activities observed.

A second particular crucial point comes from a few studies indicating possible spectrophotometric measurements of GST in serum (Habdous et al. 2002). A careful re-examination of the procedure demonstrated that the level of the enzyme calculated using this method turned out to be much higher than that found using RIA or ELISA procedures. Actually pH-dependent artifacts strongly affect the results. In fact, the spectral changes previously interpreted as a measure of GST activity are mainly due to an increase of the spontaneous reaction between the two substrates (Fabrini et al. 2012a).

In conclusion, the GST activity in normal serum cannot be correctly determined with this spectrophotometric assay because of the very low enzyme concentration and the pH-dependent artifacts. Despite this re-examination, some researchers have continued to use the Habdous procedure, thus providing an unspecified number of erroneous results.

GSTP1-1 Polymorphism

The Pi class contains only one enzyme, the GSTP1-1 (one gene, *GSTP1*); four alleles in human genetic polymorphisms (*GSTP*A*, *GSTP*B*, *GSTP*C*, and *GSTP*D*) and 11 different alterations in gene or in nucleotides like A313, C341, C555, G313, T555, T341 and others. The well-characterized polymorphism of amino acid 105 substitution (i.e., Ile105Val) affects the substrate-binding site, resulting in the reduction of substrate affinity (Hayes et al. 2005).

GSTP1-1: An Enzyme Dedicated to Biomedical Applications

In many studies, it was observed that GSTP1-1 is hyper-expressed in tumor cells and closely involved in carcinogenesis, tumor formation, and resistance toward chemotherapy (Aliya et al. 2003; Chen et al. 2013; Kural et al. 2019; Lee et al. 2014; Parker et al. 2017; Sawers et al. 2014).

This enzyme apparently fulfills a protective role for tumor cells through weakening the efficacy of chemotherapeutic agents by causing their extrusion from cell and by inhibiting the MAPK pathway. Thus, a number of anticancer drugs have been designed to inhibit GSTP1-1 in order to increase the efficiency of chemotherapy. They include ethacrynic acid and its analogs (Burg et al. 2002; Li et al. 2012), TLK117 and TLK199 (Mahadevan and Sutton 2015; Raza et al. 2009), and NBDHEX (Ricci et al. 2005; Rotili et al. 2015).

Hyper-Expression of GSTP1-1

The levels of GSTP1-1 as well as other cytosolic GSTs (e.g., alpha, mu, and theta) are up-regulated by exogenous compounds (Higgins and Hayes 2011). In particular, a study on rat showed that expression of GSTP1-1 was induced preferentially by polycyclic aromatic hydrocarbons, phenolic antioxidants, and thiol-reactive chemopreventive agents. Rats treated with a diet enriched with different xenobiotics showed induction of liver GST subunits as determined by immunoblotting. GSTP1-1 was more expressed by rats in which ethoxyquin, butylated hydroxyanisole, coumarin, diethyl maleate, benzyl isothiocyanate, β-naphthoflavone, and *trans*-stilbene oxide were administered (Higgins and Hayes 2011).

Tissue Distribution of GSTP1-1 in Humans

The importance of the GSTPi class is underlined by the widespread tissue distribution of this enzyme. GSTP1-1 expression was quantified in human tissues by immunohistochemical staining studies, RNA sequence database, HPLC, mass spectrometry, and immunoblotting. Interestingly, GSTPi is widely diffused in all human tissues, but it is mainly present in the bladder, skin, lung, small intestine, kidney,

ovary, placenta, and thyroid, whereas in liver only, there is a rather small amount of this GST class (Buratti et al. 2021; Dhanani and Awasthi 2007; Rowe et al. 1997; Terrier et al. 1990). GSTP1-1 is the most abundant GST in erythrocytes, representing the 95% of the entire GST pool.

GSTP1-1 in Human Pathologies

GSTP1-1 hyper-expression is associated with a variety of human pathologies. As mentioned above, GSTPi levels increase in tumors of non-small cell lung, breast, colon, and pancreas (Bocedi et al. 2019). Moreover, the GSTPi is overexpressed in tumor cell lines resistant to chemotherapy treatments. Expression of GSTPi is also relevant in benign prostate, prostatic intraepithelial neoplasia, and prostatic adenocarcinoma (Chatterjee and Gupta 2018). An increased activity of GSTP1-1 was described in the oral mucosa in which the development of the squamous cell carcinoma is active (Chatterjee and Gupta 2018). The involvement of GSTP1-1 in liver diseases is only definite in animal models like rats for hepatocellular carcinoma and liver fibrosis (Bocedi et al. 2019). The hyperbilirubinemia is also characterized by an increased level of the erythrocyte GSTP1-1 in young human patients (Carmagnol et al. 1981). The GSTP1-1 expression is also important in neurodegenerative disorders; in Alzheimer's and Parkinson's diseases and other psychiatric disorders like autism and schizophrenia, a linkage between the levels of GSTP1-1 and the severity of the pathology has been reported recently (Bocedi et al. 2019).

Pathologies like autoimmune diseases (e.g., scleroderma, systemic lupus erythematosus, and rheumatoid arthritis) are often characterized by systemic organ damages and are possibly due to exposure to toxins, as observed in industrialized countries with a high pollution threshold. Notably, GSTP1-1 is hyper-expressed in scleroderma, and the expression correlates with the severity of this disease (Bocedi et al. 2019; Chikezie 2015).

The most studied pathologies involving systemic toxicological problems are the kidney diseases, in which this organ, which filters and cleans the blood from circulating toxins, is somehow damaged (Bocedi et al. 2016b).

The e-GST has been studied extensively in correlation with kidney diseases. The first studies that reported an unusual overexpression of e-GST in nephropathic patients were made on newborns with hyperbilirubinemia and in patients in hemodialysis (Carmagnol et al. 1981; Galli et al. 1999). More recently, many studies have confirmed that e-GST levels in red blood cells (RBCs) increase in chronic kidney diseases (CKD), in patients in pre-dialysis under conservative therapy, and also in hemodialysis. Thus, the enzymatic expression of e-GST is a useful biomarker to verify the blood toxicity in CKD patients; the e-GST level is not influenced by vitamin E supplementation or erythropoietin therapy but only by circulating toxins (e.g., high-molecular-weight toxins, protein-bound toxins). The e-GST expression varies in patients treated with pure diffusive and convective dialytic procedures and increases linearly along the stages of kidney disease progression. An enhancement

was also found in kidney transplanted patients (Bocedi et al. 2016b, 2018; Dessì et al. 2012).

The e-GST level may also be used to monitor the systemic toxicity in healthy organisms not affected by pathologies but only subject to chronic or acute exposure to dangerous compounds (Bocedi et al. 2019). In the following paragraphs, the application primarily of e-GST as an environmental biomarker is reported for humans, mammalians, fishes, and other animal species.

e-GST as a Long-Term as Well as a Short-Term Biomarker of Blood Toxicity

e-GST is an atypical biomarker because the exposure of a living organism to toxic and dangerous compounds may produce either an increase or a decrease in activity. Many toxins cause an overexpression of e-GST without interfering with the catalytic mechanism of this enzyme. In this case, an enhancement in activity is always observed. Importantly, an increase in activity should always be referred to an increase in e-GST levels and not to the presence of some hypothetic enzyme activators never described before (Bocedi et al. 2016b; Galli et al. 1999). The maximum effect for this overexpression is expected after about 2–5 months of chronic exposure to the toxin. In fact, this corresponds to the life span of erythrocytes in mammals, and e-GST is only synthetized during erythropoiesis and not later because mature erythrocytes do not synthetize de novo proteins. In this way, e-GST appears as a biomarker of chronic exposure to toxins, which resembles the behavior of glycated hemoglobin in the retrospective monitoring of blood glucose. An increase in activity observed after shorter exposition times probably only represents a qualitative underestimated indication and not a precise quantitative value.

The presence of toxins that display inhibitory interference toward the catalytic mechanism of e-GST causes a decrease in activity that can be observed even after a very short exposition time. In this case, e-GST may represent a biomarker of acute toxicity.

Human e-GST as Biomarker in Environmental Toxicology

The normal values of e-GST expressed in IU/gHb varies from two in dogs to six to seven in humans (Bocedi et al. 2016b; Kurata et al. 1993). Higher values of 10, 15, and 20 are found in hamster, rhesus monkey, and cat, respectively (Kurata et al. 1993). Moreover, the RBC life spans in different mammalian species are 51 days for mice, 67 for rats, 150 for sheep and humans, and 175 for cattle (Kurata et al. 1993). A careful examination of many studies on the effects of environmental pollutants on human e-GST is reported below and in Fig. 3.

An interesting study on the contamination produced by an electric power plant compared the e-GST activity in three groups of persons: one group was only indirectly exposed (working at the office), a second group was directly exposed

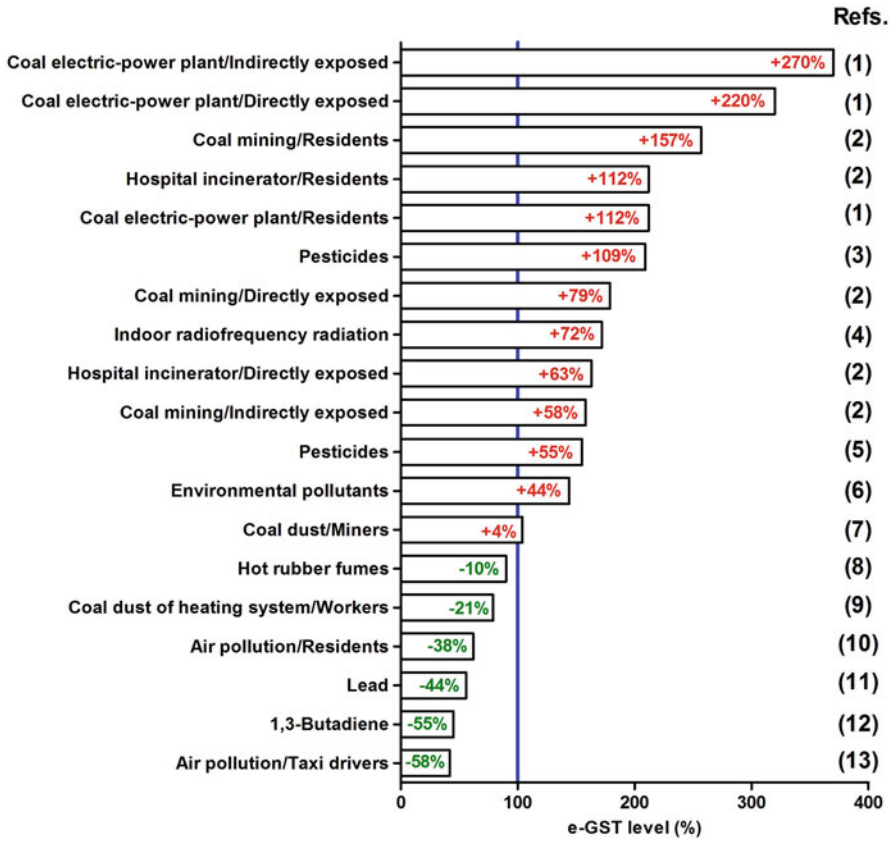

Fig. 3 Effect of toxins on the human e-GST activity as reported in several studies. Percentage change of e-GST activity (abscissa axis) in subjects exposed to various pollutants (ordinate axis). Experimental data from different studies were obtained from tables or digitalizing graphs and then calculating the percentage values with respect to the control group present in each study. The blue line represents the control value (100%). The percentage increase of the e-GST level is shown in red, while its decrease is shown in green. (Data derived from the following references: (1) Possamai et al. (2010), (2) Wilhelm Filho et al. (2010), (3) Sharma et al. (2013), (4) Akkam et al. (2020), (5) Dutta et al. (2019), (6) Fabrini et al. (2012b), (7) Schins et al. (1997), (8) Kilpikari and Savolainen (1984), (9) Tuluce et al. (2011), (10) Almutairi et al. (2020), (11) Dobrakowski et al. (2016), (12) Primavera et al. (2008), and (13) Brucker et al. (2013))

(working at the burning area), and the third group was composed of residents living near the power plant (residents). The levels of e-GST compared to the control group (100%) were +270% for indirect exposure, +220% for direct exposure, and +112% for residents, respectively (Possamai et al. 2010) (Fig. 3). This somewhat surprising result (the indirect exposure causing more damage than direct exposure) might reflect the level of protection applied. Probably, the workers who are directly exposed to dust and smoke have a better protection (filters) than office workers.

Another study, made in the Brazilian district of Santa Catarina demonstrated that contaminants in the environment such as heavy metals and dioxins, produced by coal mining and hospital waste incineration, had an impact on the local population. Compared to a control group living in an uncontaminated area (defined as 100%), the e-GST values were +157% for residents living in the vicinity of the mine, +79% for miners directly exposed underground, and +58% for miners indirectly exposed by working on the surface. Furthermore, the e-GST values for residents living near the incineration site and for the workers in the same plant were +112% and +63%, respectively (Wilhelm Filho et al. 2010) (Fig. 3). These results suggest that the residents near the mine are more contaminated than the workers; probably, the workers were protected by the use of safety equipment (filters).

The pesticides represent a second category of pollutants that are spread in the environment. Studies on agriculturist in the North India and the Bengal regions reported that a widespread use of organophosphorous compounds and carbamate pesticides has altered the levels of e-GST. Today, the intensive use of agrochemicals to protect tea plantations and other crops from insects is an important environmental problem in that part of the world. As a consequence, the e-GST levels in agricultural workers were increased by up to 109% in Northern Indian subjects with respect to a control group (Sharma et al. 2013) and a +55% increase has been measured for Bengalese workers (Dutta et al. 2019) (Fig. 3).

A new form of pollution typical of the contemporary society comes from the daily use of electric devices. A study conducted in the Middle Eastern region of Jordan revealed an increase in the e-GST level of +72% (compared to a control group) in subjects exposed to electromagnetic pollution generated by cell phone towers (Akkam et al. 2020) (Fig. 3).

An important Italian study of the contribution of e-GST as a biomarker of pollution in a geographical region with cities, villages, industrial sites, and landfills was made on 500 healthy volunteers living and working in the Sacco river valley, a region south of Rome, which is polluted by illegal industrial dumps. Eight areas were tested, and in six of them, an increased e-GST level was observed, spanning from 18% to 44% higher than the control group. The highest values corresponded to the most polluted areas as reported in Fig. 3 (Fabrini et al. 2012b).

The e-GST has been tested as possible biomarker for subjects exposed to industrial toxicants. However, in a study on workers exposed to hot rubber fumes, it was found that the e-GST activity actually decreased significantly compared to the untreated control (Kilpikari and Savolainen 1984).

Other studies were performed on subjects employed in industrial sites or in mines. A modest increase in e-GST (+4%), close to control value, was observed for 66 coal miners not affected by coal workers' pneumoconiosis, chronic bronchitis, and lung function decrease (Schins et al. 1997). A second contribution comes from a study on workers exposed to furnace coal dust particles derived from a central heating system; this study reported a value of −21% below the control group (Tuluce et al. 2011) (Fig. 3). A group of 36 males employed in periodic maintenance of blast furnaces, and thus occupationally exposed to lead for around 40 days, showed a decreased level of e-GST of −44% below the control group (Dobrakowski et al. 2016) (Fig. 3).

Thus, it appears that prolonged exposition to blast furnaces, even for maintenance, has a relevant inhibitory effect on e-GST.

Petrochemical workers exposed to low doses of 1,3-butadiene (an oxidizing compound that contaminates the air of the industrial site) showed an impaired e-GST activity, resulting in an inhibition of −55% with respect to the control (Primavera et al. 2008) (Fig. 3).

Air pollution is one of the major problems in great urban agglomerates. People who live in towns or metropolis are exposed to a complex mixture of particulate matters (PMs), ozone, hydrogen sulfide, carbon monoxide, nitrogen dioxide, ammonia, methane, nitrogen oxides, sulfur dioxide, carbon dioxide, and non-methane hydrocarbon.

Two recent studies reported the inhibition of e-GST(−38% below the control) in the resident population of a town in Kuwait. The inhabitants are exposed mainly to PM_{10} and sulfur dioxide (Almutairi et al. 2020). The taxi drivers in Porto Alegre (Rio Grande do Sul, Brazil) are instead daily exposed to $PM_{2.5}$ polycyclic aromatic hydrocarbons (benzo[a]pyrene), corresponding to −58% below the control (Brucker et al. 2013) (Fig. 3).

Mammalian e-GST as Biomarker in Environmental Toxicology

The e-GST was considered a reliable biomarker in farm animals exposed to environmental pollutants and in laboratory animal models like rat and mice. The Sacco river valley (Italy) is a highly polluted region due to illegal industrial dumping as reported above, and it represents a sort of extended open laboratory to experiment possible biomarkers. In fact, e-GST was found to be overexpressed in cows reared in farms located in this polluted area (+68%) compared to the cows reared in unpolluted areas (Bocedi et al. 2016c). Very recently, it was observed that ewes are animals involved in environmental surveillance. The ewes reared in the contaminated area express higher e-GST levels (+44%) with respect to the ewes in uncontaminated area (Bocedi et al. 2022) (Fig. 4).

Aflatoxins are contaminants in food and feed products for humans and animals. Rats fed on rice contaminated with four types of aflatoxins (B1, B2, G1, and G2) at different percentages showed an e-GST value +21% higher compared to rats fed with basal diet without aflatoxins (Yaman et al. 2016) (Fig. 4). Conversely, in a more recent study, rats were exposed to pure aflatoxin B1 added to a protein diet (normal or low-protein diet) causing a decrease of −46% e-GST(−50% in low-protein diet) below the two control groups (Rotimi et al. 2018) (Fig. 4). A possible explanation of these contradictory results may be that the inhibitory effect of B1 is compensated and even overwhelmed by an induced overexpression of e-GST by other aflatoxins.

Other important contaminants are the arsenic derivatives, and in many case, they persist in the environment especially in countries without government laws that prevent indiscriminate dumping of heavy metals. Sodium arsenite was supplied in water to rats for 4 weeks at three different doses: 10, 20, and 40 mg/kg/day. The e-GST values were very similar for the three treatments, and a calculated average

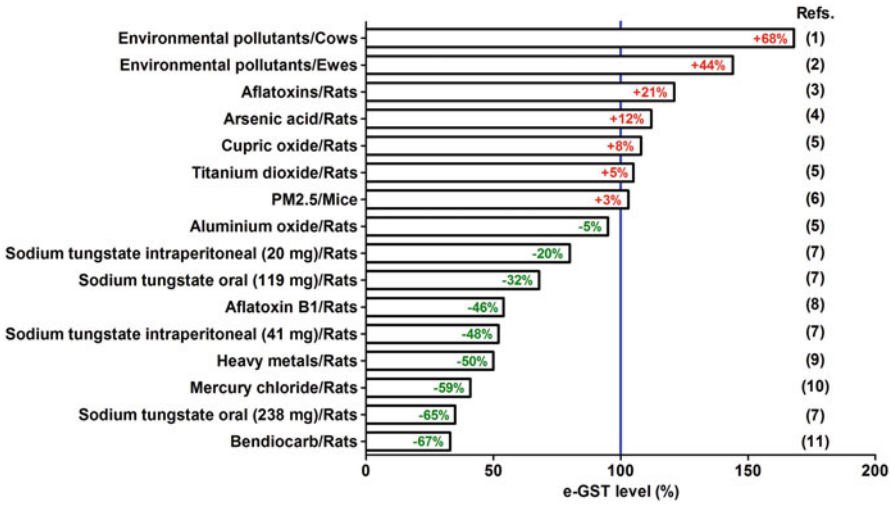

Fig. 4 Effect of toxins on the mammalian e-GST activity as reported in several studies. Percentage change of e-GST activity (abscissa axis) in animals exposed to various pollutants (ordinate axis). Experimental data from different studies were obtained from tables or digitalizing graphs and then calculating the percentage values with respect to the control group present in each study. The blue line represents the control value (100%). The percentage increase of the e-GST level is shown in red, while its decrease is shown in green. (Data derived from the following references: (1) Bocedi et al. (2016c), (2) Bocedi et al. (2022), (3) Yaman et al. (2016), (4) Oyagbemi et al. (2017), (5) Canli et al. (2017), (6) de Oliveira et al. (2018), (7) Sachdeva et al. (2013), (8) Rotimi et al. (2018), (9) Ebokaiwe et al. (2018), (10) Temel and Taysi (2019), and (11) Apaydın et al. (2018))

value of +12% with respect to untreated rats (control group) is reported (Oyagbemi et al. 2017) (Fig. 4). This probably represents an underestimate incremental value as determined only after 4 weeks of exposition.

In an another study, rats were fed with oral supplementation of metal derivatives (Al_2O_3, CuO, and TiO_2 nanoparticles), and after 14 days, e-GST level was measured. Three doses for each nanoparticles were administered, and the three e-GST average values were calculated. Cupric oxide showed +8%, titanium dioxide +5%, and aluminum oxide −5% values very close to control group (Canli et al. 2017) (Fig. 4). Also in this case, the modest variation of e-GST may be caused by a very short time of exposition compared to the life span of the rat erythrocytes.

The most widespread air pollutants are PMs; researchers exposed old male mice to ultrafine $PM_{2.5}$ containing polycyclic aromatic hydrocarbons for 5 days/week and for a total of 3 months. The value of e-GST after this exposition differs for only +3% with respect to control (de Oliveira et al. 2018) (Fig. 4).

Tungsten is present in industrial and military manufacturing, becoming during the last years a new contaminant. Male rats were administered for 14 days with an amount of 119 and 238 mg/kg of sodium tungstate orally or with 20 and 41 mg/kg via intraperitoneal route. Interestingly, an inhibitory effect on e-GST was found for

sodium tungstate correlated with the administered quantities of this compound. e-GST values were −20% and −48% for 20 and 41 mg/kg of intraperitoneal sodium tungstate and −32% and −65% for 119 and 238 mg/kg for oral doses (Sachdeva et al. 2013) (Fig. 4).

One of the greatest problems in the field of environmental pollution is represented by the contamination of potable water sources by anthropogenic and geological activities; in particular, water sources are subjected to heavy metals' presence, becoming a first line of exposure to dangerous compounds. Six groups of rats (one was the control group) were fed with borehole water from five different locations. Except the control group, the water from boreholes was polluted and gave a similar effect on e-GST, i.e., an e-GST average levels of −50% with respect to the control group (Ebokaiwe et al. 2018) (Fig. 4).

The mercury is the most dangerous among heavy metals. The exposure to mercury and mercury derivatives may permanently damage many organs like the brain and kidneys and cause cancer diseases. Male rats were fed for 10 days with mercury chloride (0.01 g/kg/day); at the end of the protocol, the e-GST level was estimated. A value of −59% with respect to the control group was found (Temel and Taysi 2019) (Fig. 4).

Bendiocarb is a pesticide of the class carbamate and is a reversible inhibitor of acetylcholinesterase activity but also of GSTP1-1. In fact, rats were fed with bendiocarb (0.8 mg/kg 1/50 LD_{50}) for an experimental period of 28 days, and at the end, the e-GST showed −67% with respect to the control (Apaydın et al. 2018) (Fig. 4).

Antibiotics are a further important class of molecules that, as reported by many studies, are inhibitors of different enzymes involved in cellular physiology. The inhibitory effect of three new generation of cephalosporins was tested on e-GST from rats. The rats were divided into different groups and injected with intraperitoneal amounts of cefazolin (single dose of 50 mg/kg), cefuroxime (single dose of 25 mg/kg), and cefoperazone sodium (single dose of 100 mg/kg). The inhibition of 50% of e-GST activity was reached in 4.65 h for cefazolin and cefoperazone sodium while in only 1.95 h for cefuroxime (Türkan et al. 2019) (Fig. 4).

e-GST in Fish Erythrocytes

Water represents 70% of the earth's surface and the major system for climate regulation, life sustainment, and the environment for great part of animal and plant life species. Moreover, water is the primary solvent of inorganic molecules, organic molecules, and bio-macromolecules. Water is also the physical storage system of pesticides, heavy metals, chemical contaminants from industrial plants, antibiotics, and many other dangerous compounds. Aquatic ecosystems are particularly affected by such substances, and the consequences are the decrease of water quality (potable water), impact on life species, and the disruption of ecological balance of the environments (Gonçalves et al. 2021). Organisms who live in water ecosystem are the first to be affected by this type of pollution, and therefore, they represent an

in vivo model for the environment surveillance. The detoxifying enzymes of fishes are the primary targets for ecotoxicological studies, and among them, the GSTP1-1 may represent an important biomarker.

Notably, two studies were conducted on e-GST from fishes. In the first one, two groups of *Carassius auratus* were exposed for 96 h to 3.9 and 7.5 mg/L of Mn^{2+}, while a third group served as control. The erythrocytes were obtained from blood samples, and e-GST was measured; e-GST levels were +156% and +68% for fishes exposed to 7.5 and 3.9 mg/L Mn^{2+} with respect to the control group (Aliko et al. 2018). These results are highly surprising because the exposition to Mn^{2+} was only for 96 h, a very short time incompatible with erythrocytes turnover.

e-GST as Biomarker in Environmental Toxicology in Other Animal Species

Besides mammals and fishes, many other species can exhibit e-GST as the major constituent of the GST pool in the erythrocytes, and thus, this enzyme could represent a possible biomarker in these species as suggested by experiments on birds.

The e-GST activity of wild birds like griffon vultures (*Gyps fulvus*) polluted with different metals spread in the environment was evaluated. The lead contamination caused an increased average value of e-GST of +26% in the birds present in the most contaminated area. This increased activity could be considered like a response of antioxidant mechanisms in griffon vultures against the higher pollutant levels (Espín et al. 2014a).

Finally, a very interesting study compared the e-GST activities from Eurasian eagle owl (*Bubo bubo*) from three areas (agricultural and rural area, industrial area, and mining area) in the south of Spain. The e-GST activity was lower in the industrial area(−13%) and in mining area(−24%) with respect to the agricultural and rural areas assumed as a sort of control group (Espín et al. 2014b). These data indicate the presence of contaminants with inhibitory activity toward e-GST.

Future Perspective and Clinical Applications

One of the major potential application of e-GST as biomarker is linked to the variations of its activity in conditions associated with chronic or acute exposure to endogenous or environmental stressors or toxins, such as environmental pollutants in urban areas, working places, or wild places affected by contaminants and polluted food or drinking water. A very peculiar characteristic of this biomarker is that the signal of dangerous contamination can be provided by both an increase and a decrease in activity depending on whether or not the contaminant is an inhibitor of e-GST. We believe that a decrease in activity is the indicator of a more dangerous contaminant as inhibiting the activity of the e-GST decreases the defense potential against toxic substances in the erythrocyte but probably also in other organs.

It must be underlined that an increase of e-GST is the consequence of chronic exposition to toxins of 2 or more months, while a decrease of e-GST activity may be also the result of a very recent contamination.

Indeed, some important advantages must be underlined:

a. The e-GST assay can be performed with a simple spectrophotometric apparatus and requires only 2–3 min/analysis. The test has a negligible cost because GSH and CDNB are inexpensive reagents.
b. The test is performed on whole blood and requires only 5–10 μL of blood.
c. The enzyme is stable for a few hours at 25 °C and 48 h at 4 °C, but the whole blood cannot be frozen as e-GST loses part of its activity.

Obviously, any alteration of e-GST cannot be referred to a specific toxin, and further chemical analyses must be made to characterize the contaminant. In this way, e-GST remembers the behavior of the white blood cells that are mainly produced in case of bacterial infection, but subsequent analyses are necessary to identify the nature of the bacterium and which organ is invaded.

It is hoped that kits for e-GST analysis on automated equipment will soon be available and supplied by pharmaceutical industries.

A clinical laboratory with automated devices to perform large-scale screening on humans and animals may be useful in the future to detect hidden contaminations in well-defined geographical area.

In conclusion, a first European laboratory exclusively dedicated to the analysis of e-GST in humans and animals will be established at the University of Rome "Tor Vergata."

Applications to Prognosis and Diseases

One of the most important planetary emergencies is the presence of toxic compounds in the soil, water, and air that can be not identified representing a "silent and hidden pollution." In fact, there are not always available simple methods that reveal precociously the presence of these dangerous compounds. The epidemiological analysis, highlighting anomalous peaks of specific pathologies, allows to point out situations of harmful contamination, although in many cases the damage to human health has already partially occurred. As described in this chapter, many studies suggest e-GST as a toxicological hazard warning "device," which may reveal early a dangerous situation for humans and animals. The fact that some kidney or systemic diseases such as sclerodermia lead to an increase in e-GST does not represent an obstacle to the use of this enzyme as a biomarker of environmental toxicity. In fact, environmental contamination causes an alteration of the activity of e-GST in several individuals or animals, as opposed to the pathology.

Mini-dictionary

- **e-GST:** Enzyme expressed in red blood cells devoted to toxic compound detoxifications.
- **Erythrocyte:** Blood cell type that contains the glutathione transferase Pi isoform.
- **Glutathione:** Tripeptide γ-L-glutamyl-L-cysteinylglycine that serves as reducing agent in biochemical reactions.
- **Glutathione transferases:** Dimeric isoenzymes encoded by three families of genes, divided into cytosolic, microsomal, and mitochondrial transferases. These enzymes catalyze nucleophilic attack by GSH on nonpolar compounds with an electrophilic carbon, nitrogen, or sulfur atom.
- **Pollutant:** Contaminant with natural or anthropogenic origin.

Key Facts of Glutathione Transferase

GSTs are isoenzymes evolutionary correlated with a common dimeric structure.
GSTPi is widely studied in humans, other mammalians, fishes, and other animal species.
GSTP1-1 is expressed in animal tissues and in particular in the erythrocytes.
e-GST is a useful biomarker for environmental toxicology because of its enzymatic properties.
e-GST is overexpressed or inhibited after chronic or acute exposure of the organism to endogenous or exogenous toxic compounds.

Summary Points

- GSTPi is a detoxifying enzyme with dimeric structure and peculiar enzymatic properties like negative cooperativity, temperature adaptation, and the formation of an inactive form due to an intramolecular disulfide bond.
- e-GST is hyper-expressed in chronic kidney diseases and other systemic diseases but even in case of exposition to toxic compounds.
- e-GST expression represents a biomarker in the retrospective assessment of the systemic exposure to environmental toxic compounds.
- e-GST activity in humans differs in subjects exposed to toxicants in working places, industrial sites, mines, and urban areas.
- e-GST activity varies with the animals feed, in contaminated areas, and in animals treated with toxic compounds.
- e-GST levels from fish erythrocytes treated with toxins are sensible to presence of dangerous compounds in water ecosystems.
- e-GST is also studied in other animal species (like birds) for ecotoxicological purposes.

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Extracellular MicroRNAs as Putative Biomarkers of Air Pollution Exposure

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Abstract

Air pollution is a global environmental and public health issue. Exposure to air pollution is associated with elevated risk of morbidity and mortality from respiratory, cardiovascular, and neurological diseases. Following exposure to air pollution, the lung and other organ systems mount a variety of physiological and pathological responses that can promote either homeostasis or disease. There is compelling evidence for epigenetic regulation of these biological responses by small, approximately 22-nucleotide-long microRNAs (miRNA). MiRNAs released from cells can act as intercellular messengers, relaying biological signals to nearby or systemic tissues or cells through posttranscriptional regulation, leading to biological changes. Because they are accessible for sampling from biological fluids, extracellular miRNAs can be leveraged as biomarkers for mechanistic investigation of air-pollution-induced health effects. In addition, specific environmental challenges or disease states present unique profiles of extracellular miRNAs, which may provide a sensitive indicator of air pollution exposure as well as disease outcomes. Thus, it is possible to utilize extracellular miRNAs as biomarkers for exposure assessment, mechanistic investigation, and disease outcome prediction of air pollution exposure. This chapter delves into the current literature and discusses miRNA measurement methods, air-pollutant-specific effects on miRNA expression, miRNA's role in air-pollution-induced health effects, and the possibility of miRNAs as markers of air pollution exposure.

Keywords

MicroRNA · Biomarker · Air pollution · Extracellular vesicle · Methodology · Particulate matter · Ozone · Nitrogen oxides · Sulfur oxides · Wildfire smoke

Abbreviations

BALF	Bronchoalveolar lavage fluid
COPD	Chronic obstructive pulmonary disease
DE	Diesel exhaust
EPA	Environmental Protection Agency
EV	Extracellular vesicle
HBE	Human bronchial epithelial cells
IL	Interleukin

miRNA	microRNA
mRNA	messenger RNA
NAAQS	National Ambient Air Quality Standards
PAH	Polycyclic Aromatic Hydrocarbon
PM	Particulate matter
TNFα	Tumor necrosis factor alpha
VOCs	Volatile organic compounds

Introduction

Air pollution is the contamination of the ambient atmosphere by any chemical, physical, or biological agents at levels that modify the natural characteristics of the atmosphere and are harmful to the human health (Manisalidis et al. 2020). Air pollutants derive from natural (e.g., volcano eruption, wildfires) and anthropogenic (e.g., motor vehicles, industrial facilities, household combustion) sources (WHO 2021b). Particulate matter (PM), ozone (O_3), nitrogen dioxide (NO_2), sulfur dioxide (SO_2), carbon monoxide (CO), and lead, which garner most public health concern, are considered as criteria air pollutants by the US Environmental Protection Agency (EPA 2017).

Exposure to ambient air pollution is a major global environmental health issue. The World Health Organization estimates that approximately 91% of the world's population breathe air at unhealthy levels of air pollutants (WHO 2021c). Exposure to air pollution is linked to increased risk of respiratory diseases such as chronic obstructive pulmonary disease (COPD), lower respiratory tract infections, and lung cancers and cardiovascular diseases such as ischemic heart disease and stroke, which are worldwide leading causes of death (WHO 2020). Exposure to ambient air pollution was estimated to be responsible for approximately 4.5 million global deaths in 2019 (Collaborators 2020). Increasing evidence also indicates an association between air pollution exposure and neurodevelopmental disorders in children (Volk et al. 2021).

Air-pollution-induced health effects are believed to be mediated through biological pathways involving oxidative stress, inflammation, and autonomic nervous system imbalance (Newby et al. 2015). The signaling cascades leading to oxidative stress and inflammation are regulated in part by epigenetic mechanisms, among which is the regulation by microRNA (miRNA) expression (Alfano et al. 2018). MiRNAs are a family of small (approximately 22 nucleotides), single-stranded, and noncoding RNAs that play an important role in posttranscriptional regulation of gene expression in cells (Zhao et al. 2019). MiRNAs can be secreted into circulating body fluids (e.g., extracellular vesicles (EVs)) in a stable and cell-free form; these extracellular miRNAs can be transported to recipient cells, passing on biological signaling information (Zhao et al. 2019). The cell-to-cell communication facilitated by circulating extracellular miRNAs makes it possible that inhalational exposure to air pollution induces adverse biological effects not limited to the exposed pulmonary cells, but also to those in the cardiovascular or nervous systems.

Because of their role as broad-acting posttranscriptional regulators, miRNAs are commonly considered as biomarkers for mechanistic investigation of disease pathophysiology. For example, alterations in specific miRNAs such as *miR-195*, *miR-199a*, *miR-214*, *miR-23a*, and *miR-24* are believed to be associated with cardiovascular diseases through biological processes including tissue remodeling, inflammation, tissue injury, and oxidative stress (Ardekani and Naeini 2010). Lung adenocarcinoma was associated with downregulated expression of *miR-124-5p*, *miR-144-5p*, *miR-143-5p*, and *miR-320a*, which target mRNA expression of enzymes for cell glycolytic metabolism (Iqbal et al. 2019). In addition to disease-related variation, extracellular miRNAs may present an expression profile that is tissue-specific. For example, exposure to nitrogen oxides (NO_x) was associated with highly expressed miRNAs such as *miR-25-3p* and *miR-107* in multiple organ systems including the lung, heart, kidney, and brain, but some other miRNAs such as *miRNA-6514-3p* are highly specific in breast tissues (Krauskopf et al. 2018). Therefore, considering recent advancements in sampling and quantitation methodologies in miRNA measurement, it is possible to utilize extracellular miRNAs as biomarkers in a cost-efficient manner to predict risk of biological effects in specific tissues or organs induced by environmental challenges.

There is an increasing interest to investigate the possibility of employing extracellular miRNAs as possible biomarkers of exposure to air pollution; however, knowledge gaps remain to assure their applicability and feasibility. In this chapter, we will review current literature on methodology and concept of miRNA measurement in air-pollutant-exposed biological samples, as well as epidemiological, clinical, *in vivo*, and *in vitro* studies focusing on the expression patterns of miRNAs affected by exposure to air pollution. We will also highlight knowledge gaps and identify future perspectives of using miRNAs as mechanistic and prognostic biomarkers of air pollution exposure.

Methods of MiRNA Measurement

Sample Types

Before miRNAs can be leveraged as biomarkers, they must be isolated from a tissue or biological fluid of interest. In the context of research on the inhalational exposure to air pollution, there are two main strategies to collect samples for *in vivo* miRNA expression analysis. In the first strategy, an investigator could collect solid tissue biopsies or brushings, which involve the physical disruption of the tissue to liberate cells or materials of interest. For example, with the aid of flexible bronchoscopy (ATS 1987), a tiny brush or forceps can be guided to sample airway cells of the lower respiratory tract. A second strategy involves using a liquid biopsy to sample fluid compartments such as the airway lining fluid or the blood. Commonly targeted fluid compartments in air pollution exposure studies include blood, sputum, and bronchoalveolar lavage fluid (BALF). Blood collections are routinely performed via venous sampling and venipuncture or using a lancet to draw capillary blood from

a finger (Ialongo and Bernardini 2016). To collect lavage fluid, a specific airway region can be rinsed with a physiological saline solution (Rose and Knox 2007). For sputum, a human subject is exposed by inhalation to nebulized hypertonic saline, which induces the expectoration of airway secretions (Nicholas and Djukanovic 2009). A drawback to collecting lavage fluid is that low-abundance analytes may be further diluted beyond the limit of detection, requiring additional methods of sample concentration. A technique was recently developed to sample the nasal epithelial lining fluid using absorbent paper strips, which helps to maintain sample concentration (Rebuli et al. 2017). In general, relative to solid tissue biopsies, liquid biopsies are easier to perform and less invasive. In addition to considering invasiveness or stress to study subjects, the choice of sampling method depends on a number of factors including cost, throughput, and cell or organ specificity (e.g., lung bronchial epithelial cells by bronchoscopy). For *in vitro* biomarker studies, the analogous sample types to liquid and solid biopsies are aspirated aqueous culture media or washes and the collection of the underlying cells, respectively (Bhowmick and Gappa-Fahlenkamp 2016).

Sample Processing

After performing one of the aforementioned methods of collection, the resulting samples can be processed for miRNA analysis immediately or stabilized for future use (e.g., cryopreservation, RNA stabilization solution). Isolation of miRNA from tissue samples may be conducted using a traditional RNA purification method such as phenol chloroform extraction (Chomczynski and Sacchi 2006). However, protocols using commercial RNA isolation kits have become increasingly popular for their safety, ease of use, and suitability for automation (Moldovan et al. 2014). Typically, RNA isolation kits are aimed at extracting all RNA species (total RNA), but there are methods available specifically focusing on miRNA extraction. Due to the wide variety of RNA isolation kits on the market, a best practice is to evaluate several candidate kits for suitability to a particular study (Witwer and Halushka 2016). An evaluation of isolation methods should also include determination of compatibility with a particular sample type and assessment of RNA sample yield and quality using methods including spectrophotometry, fluorescent-dye-based assays, or electrophoresis. Additional considerations should be made to assess the impact of extraction bias (e.g., toward short vs. long RNA or transcripts with high or low GC content), which can be influenced by both miRNA's primary and secondary structure and stability (Wright et al. 2020).

Sampling MiRNAs from Different Biological Contexts

MiRNAs are often sampled from the entire contents of a biopsy sample for practical considerations such as optimization in throughput, cost, and labor. However, this obscures much of the biological context of the miRNA because, for instance, a

miRNA may be intracellular and expressed by a specific cell type or it may be extracellular and carried by a macromolecular complex (e.g., Argonaute 2 (AGO2), EVs, or lipoproteins) (Geekiyanage et al. 2020). Performing additional steps to evaluate miRNA in these contexts may provide information on their biological function or directly identify target cells or organs where specific miRNAs could have functional effects. By using flow cytometry and fluorescence-activated cell sorting (FACS) on induced sputum or BALF samples, for example, a researcher could isolate inflammatory leukocyte populations (e.g., neutrophils, eosinophils, or macrophages) and specifically analyze miRNA expression in these cells (Lay et al. 2011).

Recently, there has been a great deal of interest in analyzing miRNA carried within circulating EVs. EVs are a heterogenous population of nanometer-sized, lipid membrane-bound particles that include apoptotic bodies, microvesicles, and exosomes (Raposo and Stoorvogel 2013). They are released from all cell types and are thought to play important roles in maintaining homeostasis through intercellular communication. After release from original cells, EVs may be taken up by neighboring or distant cells, where their contents, including miRNAs, may induce transcriptional and functional changes of the target cells (Raposo and Stoorvogel 2013). EV-specific miRNAs are currently isolated from samples by several different methods. Ultracentrifugation is perhaps the gold standard technique although co-isolation of contaminants with the EV output may occur (Crescitelli et al. 2021). More recently, commercially available precipitation, column purification, and ultrafiltration kits have become popular (Brennan et al. 2020). After isolation, EVs are identified by their size (50–1000 nM) and the presence of specific EV-associated markers such as surface or cytosolic proteins (Thery et al. 2018). It is important to note that the field of EV research and the classification criteria for sub-types and cells of origin are undergoing a period of rapid advancement and change. Nonetheless, EVs pose an exciting possibility in that their marker proteins could be leveraged to identify their cell-type origins and trace their destination. As such, collection of EV miRNA and further analysis of associated EV protein or lipid markers might therefore improve the specificity and predictive power of EV miRNAs as biomarkers (Moller and Lobb 2020).

Expression Analysis

There are several approaches to measure the abundance of specific miRNA transcripts. For miRNA biomarker studies, the most commonly used techniques include quantitative real-time PCR (qPCR), droplet digital PCR (ddPCR), microarray, and next-generation sequencing. A host of specially designed qPCR and ddPCR assays are commercially available for the detection of miRNA isolated from biological specimens (Forero et al. 2019). Both qPCR and ddPCR can be quantitative and highly sensitive but are limited in the number of transcripts that can be analyzed at one time. PCR-based methods are highly sensitive because they involve amplification of transcripts prior to detection with fluorescent dyes or probes. Unfortunately,

amplification can also introduce bias and variability between assays (Acinas et al. 2005). Increased throughput can be achieved using microarrays or RNA sequencing; however, the increasing availability and decreased costs of RNA sequencing are making this method preferable over microarrays. Indeed, sequencing provides the highest throughput and, unlike microarrays or other techniques, enables unbiased genome-wide profiling of the miRNA transcripts and the discovery of novel miRNAs and their variants (Pritchard et al. 2012). Unsurprisingly, this power of detection remains at a relatively high cost, requiring cDNA library preparation, sequencing instruments, and specialized bioinformatics and statistical analysis pipelines. More novel expression analysis platforms, such as NanoString nCounter (Malkov et al. 2009), have been developed that can analyze hundreds to thousands of miRNAs using hybridization of molecular barcodes. Importantly, these platforms require a single step and do not require amplification or cDNA library preparation and have lower cost than sequencing. The high throughput and reproducibility of these techniques make them well suited for biomarker studies, which seek to analyze many samples from diverse populations.

Drawing biological insights from larger-scale or global miRNA expression profiling experiments, with data on hundreds to thousands of transcripts, has become increasingly tractable through new bioinformatic tools. Although they vary in exact steps, basic pipelines for analysis of miRNA expression from an RNA sequencing run consist of 1) quality control/quality assurance of reads, 2) annotation of reads as specific miRNAs by alignment to a genome, 3) quantification of the number of specific miRNA transcripts present in a sample, and 4) statistical differential expression analysis (Chen et al. 2019). In addition to quantification of specific miRNA and statistical comparisons, bioinformatic algorithms are available to predict the biological impact of changes in miRNA expression. These algorithms typically involve miRNA-sequence-based, gene-target predictions followed by gene set or pathway enrichment analyses on a list of target genes (Garcia-Moreno and Carmona-Saez 2020). A graphical summary of methodology for miRNA measurement and analysis can be found in Fig. 1. In the following sections, we will discuss studies on the effects of the air pollution exposure on miRNA profiles that successfully employed combinations of the aforementioned techniques for sample collection, processing, expression analysis, and pathway analysis.

Effects of Air Pollution on MiRNA Expression

MiRNA regulation is one of the epigenetic mechanisms controlling gene expression, leading to changes in the phenotypic translation due to environmental stressors (Finicelli et al. 2020). It is possible that exposure to air pollution induces health effects through transcriptional alterations mediated by miRNAs. As shown in Fig. 2, epithelial cell lining in the respiratory airways releases mature miRNAs that participate in posttranscriptional control of target mRNAs and maintain homeostatic signaling cascades in targeted cells and tissues. As the first line of defense, these respiratory cells respond to the exposure of air pollutants and release miRNAs with

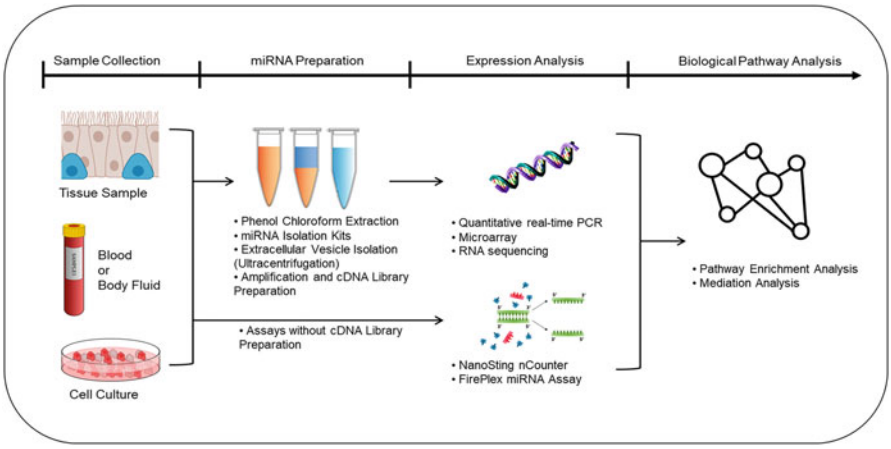

Fig. 1 Schematic illustration showing the methods of miRNA isolation and measurement in biological samples

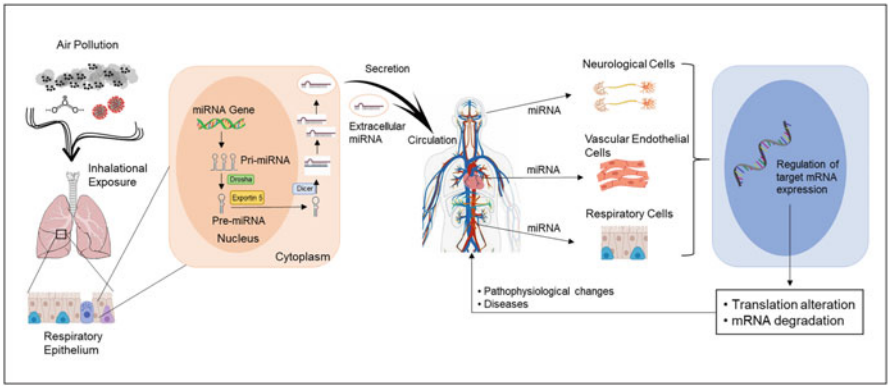

Fig. 2 Pathway illustration of air-pollution-induced health effects through alteration in miRNA expression. Epithelial cells in the respiratory airway exposed to air pollutants release extracellular miRNAs. The changed miRNA profile from air pollution exposure conveys their signals to adjacent pulmonary cells or distant cardiovascular or nervous cells. The altered miRNAs regulate mRNA expression in the target cells, resulting in phenotypic changes manifested as health effects

altered expression levels to cells *in situ* or systemically through circulation. Changes in the extracellular miRNA profile could convey biological signals to adjacent pulmonary cells or to distant cells, for example, cells in the cardiovascular and nervous system, leading to alterations in mRNA expression at these target sites. Thus, transcriptional changes induced by miRNAs can ultimately result in phenotypic changes that manifest as health effects. Here, we delve into the current literature including epidemiological, clinical, *in vivo*, and *in vitro* studies to explore whether exposure to air pollutants affects expression profiles of extracellular

miRNAs. Additionally, we examined studies that have specifically investigated the pathways by which miRNAs mediate air-pollution-exposure-induced health effects.

Effects of PM Exposure

Particulate matter is categorized as coarse (PM_{10}), fine ($PM_{2.5}$), and ultrafine ($PM_{0.1}$) particles based on their aerodynamic diameters in microns (e.g., $PM_{2.5}$ represents particles <2.5 μM). Due to their small size, airborne $PM_{2.5}$ and $PM_{0.1}$ are of most concern since they can reach deeper part of the respiratory airways such as bronchioles and alveoli, inducing inflammatory and fibrotic injuries to the lung tissues (Kim et al. 2015). Extensive literature has shown that exposure to PM is associated with increased risk of respiratory and cardiovascular diseases (Kim et al. 2015). The International Agency for Research on Cancer (IARC) has already classified PM from outdoor sources as carcinogenic and mutagenic to human beings (Loomis et al. 2013).

Epidemiological studies indicate that circulating miRNAs may play an important role in the pathogenesis of PM-exposure-induced health effects. An epidemiological study reported a differential expression of *miR-21*, *miR-222*, and *miR-146a* in peripheral blood leukocytes of the steel plant workers exposed to different levels of metal-rich PM before and after their work shift (Bollati et al. 2010). The same group further isolated microvesicles (MV) from the plasma samples of the steel plant workers and found elevated expression of *miR-128* and *miR-302c* in plasma MV after 3 days of workplace PM exposure (Bollati et al. 2015). Another epidemiological study showed that short-term exposure to ambient PM_{10} was associated with altered expression of circulating miRNAs including *miR-1537*, *miR-548*, *miR-200a*, *miR-520-3p*, *miR-155*, and *miR-501-3p* between truck drivers and office workers (Hou et al. 2016). Fossati and colleagues reported that exposure to ambient air pollution, including $PM_{2.5}$, black carbon, organic carbon, and sulfates, downregulated the expression of *miR-1*, *miR-126*, *miR-135a*, *miR-146a*, *miR-155*, *miR-21*, *miR-222*, and *miR-9* in blood leukocytes in 153 elderly male participants in the Normative Aging Study (Fossati et al. 2014). The same group later expanded the sample size to 533 participants with repeated measurements and showed an association between black carbon and changes in *miR-9* and *miR-96* expression, leading to variations in single-nucleotide polymorphisms (Colicino et al. 2016). Liu and colleagues observed that $PM_{2.5}$ exposure (based on monitoring station data) was associated with upregulation of plasma *miR-182* and *miR-185* among 109 participants, possibly linked to lung carcinogenesis (Liu et al. 2015). In addition, ten participants exposed to the highest metal-rich PM showed increases in expression of circulating *miR-29a*, *miR-146a*, and *miR-421*, which are involved with inflammatory gene expression that may result in respiratory and cardiovascular pathologies (Motta et al. 2013).

In vivo experiments also investigated the effects of inhalation exposure to PM on the miRNA profiles of rodent tissues. Inhalation exposure to 3.5 mg/m^3 of synthetic $PM_{2.5}$ for 4 h significantly decreased the expression of 24 cardiac miRNAs,

including *miR-1*, *miR-21*, *miR-26a*, *miR-133a*, and *miR-145a*, in spontaneously hypertensive rats, indicating a possible mechanism of miRNA regulation in the PM-induced cardiac effects (Farraj et al. 2011). Bourdon and colleagues exposed C57BL/6 mice to 0.162 mg carbon black nanoparticles via intratracheal instillation resulting in downregulation of *miR-135b*, *miR-146a*, *miR-146b*, and *miR-21* in the mouse lung but not in the cardiac tissue (Bourdon et al. 2012, 2013). It is noteworthy that PM compositions vary by location, hence, their potential effects on circulating miRNAs. A study compared the toxicity of PMs from different cities around the world (i.e., Zurich, Beijing, San Francisco, and Johannesburg) demonstrating that city-specific PMs induce different health effects by selectively affecting miRNA profiles in rats (Chen et al. 2020b). These researchers also suggested that upregulation of certain miRNAs, such as *miR-21* and *miR-125b*, may mitigate the health effects of PM through transcriptional regulation of functional genes (Chen et al. 2020b). Some *in vitro* studies have also shown the impacts of PM challenge on the miRNA expression levels. For example, human bronchial epithelial (HBE) cells exposed to 50 μg/mL $PM_{2.5}$ showed altered expression of *miR-145-5p*, *miR-25-3p*, and *miR-215-5p*, which could serve as potential biomarkers for $PM_{2.5}$-induced carcinogenicity (Cai et al. 2021).

Pathway enrichment analyses have been employed to predict molecular pathways and their downstream targets in mRNAs and proteins and their biological effects based on the altered miRNA profiles by PM exposure. For example, PM exposure-associated miRNAs have been identified to regulate mRNAs and proteins involved in oxidative stress and inflammatory pathways (Bhargava et al. 2019), mitogen-activated protein kinase and nerve growth factor signaling (Bollati et al. 2010), viral immune responses (Hou et al. 2016), neurological development and cognitive function (Chao et al. 2017), and vascular endothelial function (Dai et al. 2017). These studies highlight the notion that exposure to PM is associated with changes in miRNA expression, which are targeting regulation of downstream pathways that may play a role in the pathogenesis of respiratory, cardiovascular, and neurological diseases.

Effects of Diesel Exhaust Exposure

Diesel exhaust (DE) consists of carbon soot, organic components, and gaseous components, including CO, NO_x, sulfur oxides (SO_x), and polycyclic aromatic hydrocarbons (PAH) (Okubo and Kuwahara 2020). DE is classified as a carcinogen to human by the World Health Organization based on sufficient evidence that exposure to DE is associated with increased risk for lung cancer (IARC 2012). Due to the complexity of its components, we reviewed the miRNA literature of DE separately from PM.

Goodson and colleagues reported a differential methylation of *miR-133* between adult mice in the filtered air group and those exposed to 300 ug/m^3 DE particles in utero, indicating an increased sensitivity to air-pollution-induced heart failure (Goodson et al. 2017). Li and colleagues reported that $APOE^{-/-}$ mice exposed to

ultrafine PM, likely from vehicular sources, had higher risk of atherosclerosis and vasculature remodeling, which were mediated through dysregulation of *miR-301b-3p* and *let-7c-1-3p* and their downstream targets such as tissue growth factor beta 1 (Li et al. 2021). An *in vitro* study assessed the miRNA profiles of primary HBE cells in response to 10 μg/cm^2 DE particle exposure for 24 h and identified that 197 out of 313 detectable miRNAs were either upregulated or downregulated by DE (Jardim et al. 2009). Among those significantly changed miRNAs, twelve, including *miR-513a-5p*, *miR-923*, *miR-26b*, and *miR-96*, were associated with tumorigenic processes based on the pathway enrichment analysis results. A major component of DE, organic compounds such as PAH may play a significant role in DE-induced miRNA alterations as indicated that PAH increased expression of *miR-155* in HUVEC cells (He et al. 2018).

Effects of Ozone Exposure

Ozone (O_3) is a highly reactive and ubiquitous ground-level air pollutant, formed from chemical reactions of molecular oxygen, NO_x, and other volatile organic compounds (VOCs) in the presence of ultraviolet light (Mudway and Kelly 2000). Exposure to O_3 is known to induce adverse health effects such as decrements in pulmonary function and exacerbation of respiratory and cardiovascular disease. Because of its significant environmental and public health impacts, O_3 is listed as one of the US EPA's criteria air pollutants for regulation, with the current National Ambient Air Quality Standards (NAAQS) for O_3 set at 70 ppb (EPA 2013a). Although the health effects of O_3 are well documented, research on miRNA in both biomarker and functional studies in the context of O_3 exposure is limited compared to other criteria air pollutants.

The first study to explore O_3-induced changes in miRNA of the respiratory tract examined the miRNA profile of sputum samples from 20 healthy human volunteers exposed to 0.4 ppm O_3 for 2 h by a microarray assay (Fry et al. 2014). This work demonstrated that acute O_3 exposure was associated with altered expression of airway miRNAs including *miR-132*, *miR-143*, *miR-145*, miR-199a, *miR-199b-5p*, *miR-222*, *miR-223*, *miR-25*, *miR-424*, and *miR-582-5p*. Among these miRNAs, *miR-143* was significantly correlated with the percent increase in sputum neutrophils, a signature inflammatory event following acute O_3 exposure. Furthermore, they also reported that the specific miRNA expression profiles varied by susceptibility to O_3-induced inflammation (so-called responders vs. nonresponders) (Fry et al. 2014).

Several hypothesis-driven, miRNA-focused studies have been conducted in animal models to investigate O_3-exposure-induced effects. Clay and colleagues demonstrated a functional role of miRNAs (e.g., *miR-149*, *miR-202*, and *miR-410*) targeting interleukin-6 (IL-6) in primary airway epithelial cultures from juvenile rhesus monkeys exposed to 0.5 ppm O_3 for 8 h per day for 5 days (Clay et al. 2014). Fuentes and colleagues identified sex differences in the profiles of lung miRNAs following acute exposure to 2 ppm O_3 (Fuentes et al. 2018), and data suggest that male-specific sex differences in lung miRNA expression may be influenced by

surfactant protein expression (Noutsios et al. 2017, 2019). These studies suggest that miRNA-based epigenetic regulation of gene expression may play an important role in the respiratory responses to acute O_3 exposure.

MiRNAs in BALF or plasma/serum have been investigated as biomarkers of O_3 exposure. For example, Snow and colleagues examined the impact of fish oil–, olive oil–, or coconut oil–enriched diets on serum miRNA expression in addition to more-traditional markers of lung injury and inflammation (e.g., BAL cells and protein) in rats following an acute O_3 exposure (Snow et al. 2018), where they did not find O_3-induced changes, but diet-related alteration in serum miRNA profile. Two recent studies demonstrated O_3-induced changes in the expression of EV miRNAs. In a targeted study evaluating the human serum EV miRNAs related to cardiovascular disease, Chen and colleagues determined that exposure to ambient O_3 was associated with changes in the expression of plasma EV *miR-150* and *miR-155* among coronary artery disease patients (Chen et al. 2020a). The other study identified O_3-concentration-dependent changes in the profile of EV miRNAs and other small RNA species in EVs isolated from BALF of mice (Smith et al. 2021). Through bioinformatic analyses, EV *miR-2137* and EV *miR-22-3p* were identified as candidate miRNA regulators of O_3-induced inflammation. Importantly, these studies represent the first investigations of air-pollution-induced changes in EV miRNAs sampled from the blood and BALF *in vivo*, elucidating the possibility of utilizing EV miRNAs as biomarkers for air pollution exposure.

Effects of NO_x Exposure

Nitrogen oxides are various nitrogenous chemical species (e.g., NO_2 and NO) that exist in the atmosphere (Schlesinger and Lippmann 2020). Ambient NO_x are generated from both terrestrial sources, such as forest fires, organic decay, and anthropogenic activities that involve vehicular and industrial combustion (Henderson et al. 2007). NO_x exposure is known to cause elevated mortality due to respiratory and cardiovascular diseases (EPA 2021). It should be noted that many adverse health effects associated with NO_x are often attributed to PM because they share primary sources of production (Ban-Weiss et al. 2008).

Epidemiological studies have shown that exposure to ambient NO_x is linked to altered expression in circulating miRNAs. In a crossover study, expression of circulating miRNAs was compared between settings of low (i.e., in a park) and high (i.e., near a busy street) levels of exposure to traffic-related air pollution, where NO_2- and NO-associated effects were assessed separately (Espín-Pérez et al. 2018; Krauskopf et al. 2018). The results indicated that exposure to higher levels of ambient NO_2 and NO was associated with increased expression in *miR-25-3p*, *miR-28-3p*, *miR-30b-5p*, *miR-107*, *miR-146-5p*, *miR-1224-5p*, and *let-7i-5p* and decreased expression in *miR-27a-5p*, *miR-133a-3p*, *miR-145-5p*, *miR-192-5p*, *miR-215-5p*, *miR-425-5p*, and *miR-433-3p*. Further pathway analysis found that many of these miRNA variations were associated with pathologies including lung

cancer, COPD, asthma, cardiovascular disease, and neurological disorders (Krauskopf et al. 2019).

Effects of SO_x Exposure

Sulfur oxides are various sulfuric chemical species that exist in gaseous form or as part of PM in the atmosphere (Lippmann 2009). The gaseous form of SO_x is dominated by sulfur dioxide (SO_2), which is primarily produced through the burning of fossil fuels from vehicular and industrial sources. The solid state of the SO_x takes the form of acidic H_2SO_4 and SO_4^{2-} salts, which can coat the surfaces of fine and ultrafine particulates (Maricq et al. 2002). Exposure to SO_x through inhalation of gaseous SO_2 or acidic particulates containing SO_4^{2-} is known to cause bronchoconstriction, specifically through increased airway resistance, decreased expiratory flow and wheezing and shortness of breath, and increased morbidity and mortality (Nadel et al. 1965).

The bronchoconstriction and exacerbation of respiratory diseases such as asthma and chronic bronchitis associated with SO_2 exposure may be partly mediated through the miRNA regulation. Inhalation exposure of C57BL/6 male mice to a mixture of "coal-burning" pollutants (i.e., 0.5 mg/m^3 SO_2 + 0.2 mg/m^3 NO_2 + 1 mg/kg $PM_{2.5}$) caused downregulation of *miR-337-5p* and *miR-338-5p* (Ji et al. 2016; Ku et al. 2017a), implicating neurodegeneration and activity of hypoxia-inducible factor 1-alpha (HIF1A), an important protein target in cancer biology, vascularization, angiogenesis, and energy metabolism (Yu et al. 2017).

Effects of Wildfire Smoke Exposure

Wildland fire has attracted much public interests due to its increased frequency and intensity of wildfire events in recent years. In addition to direct life and property threat from wildfires, smoke generated from periods of wildfire contributes to ambient air pollution, leading to significant adverse health impacts (Cascio 2018; Chen et al. 2021). Depending on the composition of the burned materials, the mixture of wildfire smoke ranges from a variety of compounds including PM, PAHs, VOCs, polychlorinated biphenyls, and other chemicals such as aldehydes, ketones, sulfides, dioxides, and heavy metals (Chen et al. 2021). $PM_{2.5}$ derived from wildfire smoke are known to cause cardiovascular events such as myocardial infarction, stroke, arrythmia, and heart failure; decrements in lung function; development of respiratory disease like asthma and COPD; and about 300,000 deaths global/year (Chen et al. 2021). Many pathologies associated with wildfire smoke exposure may involve the regulation of altered expressions and functionalities of miRNAs. It is worth noting that in the epidemiological and experimental settings, wood smoke and/or biomass smoke are common surrogates to wildfire smoke for investigation of its biological effects.

Household usage of wood or charcoal combustion for heating and cooking resulted in higher susceptibility to esophageal squamous cell carcinoma, partly through the upregulation of *miR-423-3p* expression in a population in South Africa (Wang et al. 2013). Similarly, women with COPD exposed to biomass smoke (i.e., 361 ± 177 h/year) showed upregulation of *miR-150-5p*, *miR-191-5p*, and *miR-223-3p* and downregulation of *miR-21-5p* and *miR-374a* (Velasco-Torres et al. 2019). In addition, a study conducted in Mexico reported that wood smoke exposure from biomass combustion elevated the expression of plasma *miR-126* and *miR-155* as well as increased urinary PAH levels in women (Ruiz-Vera et al. 2019). These altered miRNAs suggest elevated risk of cardiovascular events from exposure to PAH in biomass smoke (Ruiz-Vera et al. 2019).

Although it is not a focus of this book chapter, secondhand cigarette smoke from combustion of tobacco products contributes to ambient air pollution in an enclosed environment. Inhalation exposure to secondhand smoke presents major risk factors for respiratory and cardiovascular diseases and cancer (Stedman 1968; WHO 2021a). Cigarette smoke exposure *in vivo* downregulates *miR-192*, which is involved in regulating activation of the oncogenes named RAS, a family of proteins involved in cell signaling in cell growth and differentiation (Izzotti et al. 2009; Xiao et al. 2018). A review article focusing on the effects of cigarette smoke on carcinogenesis-related miRNA profiles suggests that miRNAs could be employed as clinical biomarkers for lung or urinary bladder cancer resulted from cigarette smoke (Fujii et al. 2018).

Air-Pollution-Induced Effects Through MiRNA Regulation

Exposure to air pollutants is associated with changes in extracellular miRNA expression, and pathway enrichment analysis has been used to predict the biological impacts of these affected miRNAs (Fig. 3). These results have established a biological scheme that exposure to ambient air pollution may induce biological effects, partly through posttranscriptional regulation of miRNAs. However, only a few studies have directly assessed the role of miRNA regulation in air-pollution-induced health effects.

A preliminary statistical method to examine the role of miRNAs in air-pollution-induced effects is to assess the statistical correlation or interaction between air pollutants and miRNAs on the targeted downstream mRNA targets or biological outcomes. One study reported that $PM_{2.5}$-induced changes in expression of *miR-146*, *miR-139*, and *miR-340* in mouse lung were correlated with interferon gamma (IFN-γ) in BALF, leading to imbalance of Th1/Th2 lymphocytes (Hou et al. 2018). Huang and colleagues found a significant interaction between miRNAs (*miR-24-3p*, *miR-27a-3p*, and *miR-320b*) and PAH exposure on heart rate variability (HRV) markers with comparisons between office workers and coke oven workers (Huang et al. 2016).

A more advanced method, mediation analyses, investigates the role of miRNA in the air pollution-health effects pathway and has been applied in several human studies. In a randomized clinical trial, $PM_{2.5}$ exposure was negatively associated

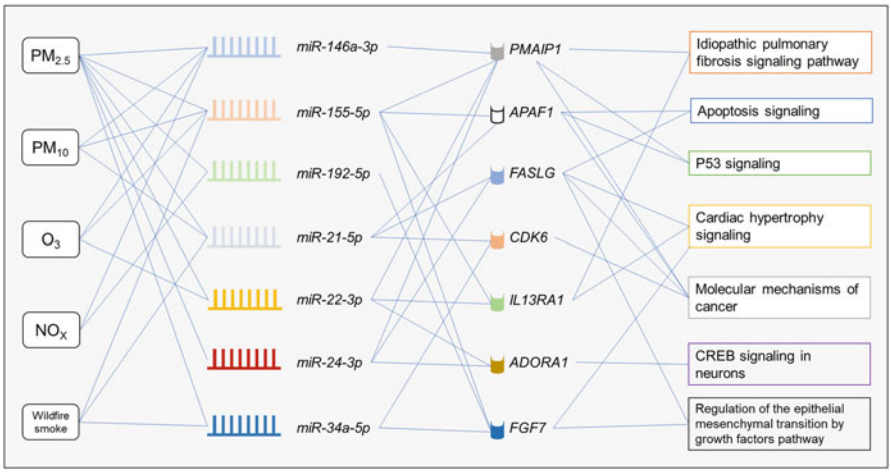

Fig. 3 Examples of the "air pollutant-miRNA-biological effects" pathway for $PM_{2.5}$, PM_{10}, O_3, NO_x, and wildfire smoke. The selected miRNA targets of air pollutant exposure were based on the literature review in this book chapter. The mRNA targets and enriched pathways were analyzed and selected from a pathway enrichment analysis using the Ingenuity Pathway Analysis (IPA) (QIAGEN, Redwood City, California). *ADORA1* adenosine A1 receptor, *APAF1* apoptotic peptidase activating factor 1, *CDK6* cyclin-dependent kinase 6, *CREB* cAMP response element-binding protein, *FASLG* Fas ligand, *FGF7* fibroblast growth factor 7, *IL13RA1* interleukin 13 receptor subunit alpha 1, *PM* particulate matter, *PMAIP1* phorbol-12-myristate-13-acetate-induced protein 1

with altered expression in *miR-21-5p*, *miR-187-3p*, *miR-146a-5p*, *miR-1-3p*, and *miR-199a-5p*, possibly mediating changes in downstream mRNAs including *IL-1*, tumor necrosis factor alpha (*TNFα*), Toll-like receptor 2 (*TLR2*), and endothelin 1 (*EDN1*) (Chen et al. 2018). Another study reported that serum levels of benzo[a] pyrene-r-7,t-8,t-9,c-10-tetrahydotetrol-albumin (BPDE-Alb) adducts, a proxy of internal dose of PM exposure, induce elevated mRNA expression of *IL-6* and *TLR2* in children, mediated by the regulation of *let-7a*, *miR-146a-5p*, and *miR-155-5p* (Li et al. 2020).

Examining specific signaling pathway may offer a causal link of miRNA mediation in air-pollution-induced adverse effects. For example, exposure to 5 mg/kg body weight $PM_{2.5}$ induced neuroinflammation and deteriorated synaptic function through the *miR-574-5p*/NFκB pathway in C57BL/6 mice (Ku et al. 2017b). Feng and colleagues reported that exposure to urban $PM_{2.5}$ induced myocardial toxicity through negative regulation of *miR-205* on the IRAK2/TRAF6/NFκB pathway (Feng et al. 2020). Another study reported that exposure of A549 cells to $PM_{2.5}$ led to cell autophagy through *miR-4516*-induced downregulation of *RPL37* and *UBA52* gene expression (Li et al. 2016). Ding and colleagues also reported that exposure to liposoluble extracts of $PM_{2.5}$ induced lipid accumulation in hepatocytes mediated by the *miR-26a*/CD36 pathway (Ding et al. 2019). These studies suggest that mediation analysis focused on miRNA may confirm the plausibility of utilizing miRNA as biomarkers for air pollution exposure.

Future Perspectives

Application

As summarized in this chapter, exposure to air pollution is associated with altered expression of extracellular miRNAs. We also discussed the role that miRNAs may mediate the air-pollution-induced health effects. Incorporating with these important findings, the following features may promote extracellular miRNAs to be considered as useful biomarkers for air pollution exposure. First, altered miRNA profiles could be unique to specific air pollutants. Second, altered miRNA profiles from air pollution exposure could also be organ- and/or tissue-specific. Third, as key regulators to mRNAs and their biological pathways and functions, certain miRNAs could provide therapeutic targets to mitigate the adverse health impacts of air pollution exposure. Therefore, a successful application of miRNAs as biomarkers for air pollution exposure may allow for air-pollutant-specific exposure assessment, target-specific health risk evaluation, and development of possible mitigation or therapeutic strategies. In addition, because miRNAs are usually encapsulated into EVs, this would allow for more targeted measurement and avoiding the contamination of other RNAs. EV miRNA-focused studies provide additional information about a miRNA's cellular source because EVs can be traced back to their releasing tissues or organs (de Miguel et al. 2020). Finally, robust and standardized methodology has been developed over the years to accurately capture the changes in miRNA profiles (Finicelli et al. 2020).

Limitation

Although miRNAs represent promising biomarkers for air pollution exposure, challenges remain to successfully validate such a role. For instance, inconsistency exists in the air-pollutant-exposure-induced miRNA profiles based on different epidemiological, clinical, *in vivo*, or *in vitro* studies. Sometimes, even contradicting results arrive when studies were compared, making it difficult to extrapolate the correct implication of the changed miRNAs. Future studies should include a more standardized strategy to allow for normalization of varied results. In addition, downstream air pollution effects mediated through miRNA are mainly predicted through pathway analysis, rather than experimental or statistical validation. This research gap needs to be addressed by utilizing more comprehensive study designs involving the whole "air pollution-miRNA-biological effects" scheme, applying more advanced statistical methodologies, and conducting functional studies in animal and *in vitro* model systems. Furthermore, the transient nature of altered extracellular miRNA expression poses a challenge to capture relevant miRNA in time to allow for their biomarker application. More research is warranted to better understand the temporal dynamics of air-pollution-induced miRNA expression to identify optimal detection window.

Conclusions

This chapter summarizes the effects of exposure to air pollution on extracellular miRNA profiles from epidemiological, clinical, *in vivo*, and *in vitro* studies. Many of the miRNA changes are believed to mediate air-pollution-induced downstream biological effects such as oxidative stress, inflammation, vascular endothelial injury, and neurocognitive dysfunction, as evidenced by the pathway investigation and mediation analysis. With increasing evidence and more standardized methodologies, extracellular miRNAs could be employed to indicate toxicity of air pollution exposure and serving as potential biomarkers and revealing therapeutic pathways.

Applications to Prognosis

In this chapter, the possibility of utilizing extracellular miRNAs as biomarkers for air pollution exposure and its associated health effects are discussed. In particular, exposure to different air pollutants can induce distinct changes in the profile of extracellular miRNAs, which allows for potential application of miRNA as exposure biomarkers. In addition, miRNA-mediated downstream health effects may be weighted to assess the disease risk of air pollutant exposure. For example, the relatively well-established link, $PM_{2.5}$-*miR-21*-cardiovascular disease, may predict the risk of cardiovascular disease attributed to $PM_{2.5}$ exposure based on the altered extracellular *miR-21* expression levels.

Applications to Other Diseases or Conditions

We mainly discussed the possibility of applying extracellular miRNAs as biomarkers for air pollution exposure and its associated biological effects. This notion is also applicable to other types of environmental challenges. Air pollution is a complex mixture of chemicals that include elemental carbon, organic carbon, heavy metals, volatile organic compounds, particulate matter, and gases (O_3, SO_2, NO_2, etc.). These components are also commonly present in other types of environmental media such as soil, water, and food. Thus, the "pollutant-miRNA-biological effects" model could also be used to assess the health effects of other types of environmental challenges in addition to air pollutants.

Mini-dictionary of Terms

- Air pollution: Contamination of indoor or outdoor air by any chemical, physical, or biological agent that modifies the natural characteristics of the atmosphere, causing respiratory, cardiovascular, and neurological health problems (WHO 2021b)

- Extracellular vesicles: Nanosized, lipid-membrane-bound vesicles released from cells into the extracellular spaces that can transport contents consisting of lipids, nucleic acids, and proteins (Doyle and Wang 2019)
- MicroRNA: Small (approximately 22 nucleotides), single-stranded, and noncoding RNAs that play an important role in posttranscriptional regulation of gene expression in cells (Zhao et al. 2019)
- Particulate matter (PM): A mixture of solid particles and liquid droplets found in the atmosphere, which are usually categorized into PM_{10} (aerodynamic diameters that are generally 10 μm and smaller, inhalable) and $PM_{2.5}$ (diameters that are generally 2.5 μm and smaller, fine inhalable) (EPA 2013b)
- Transcriptional regulation: A biological process that cells regulate the conversion of DNA to RNA, thus affecting the gene activity in responding to a variety of intra- and extracellular signals to coordinate cellular activity (Casamassimi and Ciccodicola 2019)

Key Facts of MicroRNA

- MiRNAs were first reported in *Caenorhabditis elegans* in the early 1990s (Lee et al. 1993).
- Primary miRNA (pri-miRNA) from the genome is cleaved by an RNase enzyme, Drosha, into hairpin intermediates, which are further cut into ~22-nucleotide miRNA/miRNA* duplexes by another enzyme named Dicer (Zhao et al. 2019).
- The miRNA strand of the duplex is incorporated into the RNA-induced silencing complex (RISC), and the miRNA* strand is released and degraded (Zhao et al. 2019).
- MiRNAs were released to the extracellular space through three pathways: passive leakage from broken cells; membrane-enclosed microvesicles; and microvesicle-free, RNA-binding, and protein-dependent pathways (Zhao et al. 2019).
- MiRNAs offer an important media for cell-to-cell communication and posttranscriptional regulation of gene expression (Finicelli et al. 2020).

Summary Points

- Exposure to air pollution is linked to alterations of miRNA profile in both the lung and circulation.
- Extracellular miRNAs may play an important role in air-pollution-induced health impacts through posttranscriptional regulation of target genes.
- Extracellular miRNAs may be leveraged as biomarkers to predict the risk of air-pollution-associated diseases.
- Extracellular miRNAs may also serve as biomarkers for prognosis of air-pollution-exposure-associated health effects.
- More research is needed to further validate the possibility of utilizing miRNAs as biomarkers of air pollution exposure.

Cross-References

► Biomarkers of $PM_{2.5}$ Exposure: Use of Metabolomics as a Platform
► Endothelial Extracellular Vesicles as Biomarkers of Toxic Molecules

Disclaimer The research described in this chapter has been reviewed by the Center for Public Health and Environmental Assessment, US EPA, and approved for publication. The contents of this chapter should not be construed to represent agency policy, nor the mention of trade names or commercial products constitutes endorsement or recommendation for use.

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Endothelial Extracellular Vesicles as Biomarkers of Toxic Molecules

22

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Abstract

Extracellular vesicles (EVs) released by endothelial cells stand out for potentially carrying biomarkers that reflect the state of the endothelium, which is particularly interesting in the investigation of kidney and cardiovascular diseases (CVD) that are closely linked to its dysfunction. Endothelial EVs can be obtained from blood samples; however, the study of these EVs is challenging since complete isolation and purification is still unrealistic. Despite this, studies have shown differences in the protein profile and miRNA content present in the circulating EVs of patients with CVD and kidney diseases. By carrying molecules with biological activity, EVs are able to modulate the response of recipient cells, which may also contribute to the pathophysiology of these diseases. Given the importance of EVs, especially those derived from the endothelium, this chapter addresses the EV formation and content in order to investigate potential biomarkers as well as its effector role in disease progression.

Keywords

Extracellular vesicles · Endothelial cells · Endothelial dysfunction · Cardiovascular diseases · Kidney diseases · Chronic kidney disease

Abbreviations

ACD	Acid citrate dextrose
ACS	Acute coronary syndromes
AFM	Atomic force microscopy
CAD	Coronary artery disease
CCL17	Chemokine ligand 17
CCS	Chronic coronary syndromes
CKD	Chronic kidney disease
COX2	Cyclooxygenase 2
Cryo-EM	Cryogenic electron microscopy
CTRC	Chymotrypsin C
CVD	Cardiovascular diseases
DLS	Dynamic light scattering
EVs	Extracellular vesicles
exRNA	Extracellular RNA
GC-MS	Gas chromatography-mass spectrometry
ICAM-1	Intercellular adhesion molecule 1
miRNA	microRNA
NTA	Nanoparticle tracking analysis

PEG	Polyethylene glycol
PMTs	Post-translational modifications
RT	Reverse transcription
SEC	Size exclusion chromatography
SEM	Scanning electron microscopy
SPR	Surface plasmon resonance
SRC	Proto-oncogene tyrosine-protein kinase SRC
STEMI	ST-elevation myocardial infarction
TEM	Transmission electron microscopy
TFF	Tangential flow filtration
TLC	Thin-layer chromatography
TNF-α	Tumor necrosis factor α
UPLC-MS/MS	Ultra-performance liquid chromatography-tandem mass spectrometry

Introduction

Extracellular vesicles (EVs) were first known as cellular waste or dust particles but now, with the research advancement, they are known to be of great importance for both normal and pathological conditions, especially due to their role in cell-to-cell communication. EVs are nano-sized membrane-enclosed structures released by several cell types, composed of a lipid bilayer, proteins, and molecular cargo from their cell of origin. However, cells exposed to an injury or stress release EVs with modifications in their composition, which makes them distinct from the EVs released under physiological conditions. These EVs may play a role in the pathogenesis of several diseases as they carry biomolecules from their cell of origin to other cells. Due to their nature, EVs are being extensively studied as biomarkers in several diseases and even as therapeutic strategies (Burger et al. 2013; Erdbrugger and Le 2016; Azevedo et al. 2021). EV markers can be identified before the onset of symptoms or physiological detection of the disease, which makes them promising candidates for early-stage disease detection (Andaluz Aguilar et al. 2020).

In recent years, EVs released by endothelial cells, known as endothelial EVs, have drawn attention due to their role in endothelial dysfunction. The endothelium acts as a key gateway in communication between blood and the cellular stroma, and dysfunction in endothelial cells has been reported to be of great importance, especially in cardiovascular and renal diseases (Mathiesen et al. 2021). EVs from endothelial origin can further activate the endothelium, inducing responses that lead to inflammation, oxidative stress, and apoptosis (Burger et al. 2012, 2016). Also, endothelial EVs can contain high levels of calcium and bone morphogenic protein-2, correlating with vascular calcification and osteogenic differentiation (Buendía et al. 2015; Favretto et al. 2019).

Considering the importance of EVs and their potential to be used as biomarkers, especially endothelium-derived EVs, this chapter addresses the types of EVs, the main isolation and analysis methods that are currently used, and the effector role and

content of EVs formed in cardiovascular and renal diseases, which are strongly related to endothelial dysfunction. Throughout this chapter, the role and molecular characteristics of endothelial EVs in pathological conditions are addressed, highlighting recent advances in their study.

EVs' Classification

EVs are classically classified according to their size and mechanism of formation. Recent studies have divided EVs into three main groups: microvesicles, exosomes, and apoptotic bodies (Fig. 1). Microvesicles are 100–1.000 nm sized vesicles generated from the outward blebbing of the plasma membrane of the cell. This mechanism is characterized by asymmetry loss of lipids of the plasma membrane, through cytoskeleton remodeling, and rearrangement of the lipids (Burger et al. 2013; Favretto et al. 2019; Mathiesen et al. 2021). This asymmetry loss can be induced by activation, stress, or cell death. Microvesicles have membrane proteins from their cell of origin, so they are said to be ideal biomarkers of tissue damage (Favretto et al. 2019; Azevedo et al. 2021). Exosomes are 40–120 nm sized vesicles and are formed by the inward budding of multivesicular endosomes, forming intraluminal vesicles and later fusing with the plasma membrane, releasing the exosomes into the extracellular space. This process can also be called "reverse endocytosis" (Johnstone et al. 1987; Nederveen et al. 2021). Exosomes contain functional microRNAs (miRNAs) and small RNAs that can be transferred to circulating cells. Exosomes can interact with recipient cells through endocytic uptake, direct fusion with the cell membrane, or adhesion to the cell surface (Nederveen et al.

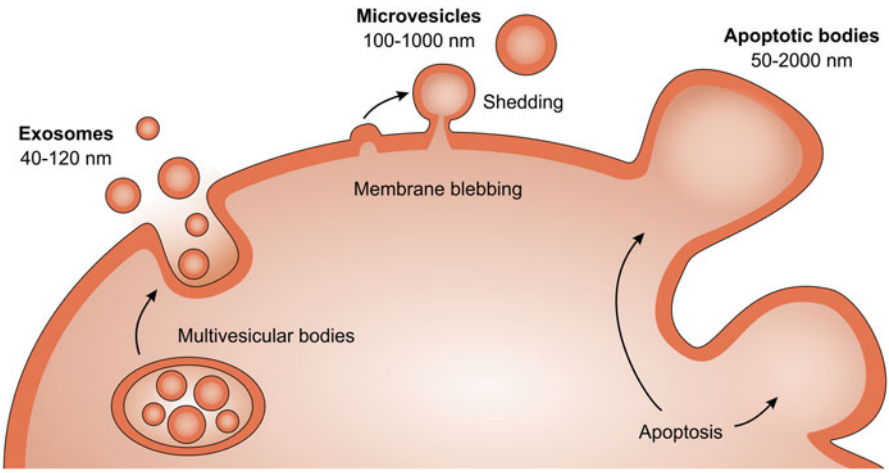

Fig. 1 Formation of extracellular vesicles. Exosomes are formed from multivesicular endosomes, microvesicles from membrane remodeling, and the apoptotic bodies from apoptotic cell death process

2021). Finally, apoptotic bodies are 50–2.000 nm sized vesicles released exclusively by cells undergoing programmed cell death (Nederveen et al. 2021).

Isolation of Endothelial EVs

Endothelial EVs are especially interesting as potential sources of biomarkers to be used in liquid biopsies. They are obtained with relative ease from blood samples by minimally invasive methods, causing no harm to the patient. Alternatively, in vitro studies isolate the EVs from the endothelial cell culture medium, in which fetal bovine serum is a concern because it can be a source of contaminants, containing EVs itself, lipoproteins, among other components.

In addition to endothelial EVs, blood has circulating EVs derived from various cell types, such as platelets, erythrocytes, leukocytes, and others. These EVs are obtained from either serum or plasma. For serum samples, it is expected to observe a higher amount of platelet-derived EVs due to platelet activation compared to plasma samples. Citrate plasma samples also have higher amounts of platelet-derived EVs than those obtained from tubes with acid citrate dextrose (ACD) and EDTA (Palviainen et al. 2020). The greater presence of platelet EVs may mask some results and should be considered in the analysis. High amounts of albumin, immunoglobulins, lipoproteins, and other abundant proteins in blood samples may also disturb the characterization of circulating EVs, especially analyses based on mass spectrometry (Pietrowska et al. 2019). The presence of these contaminants in the EV sample could mask the data and make the analysis of biomarkers difficult. Another factor related to blood samples is the viscosity, which can also interfere with the isolation of circulating EVs. To avoid this issue, it is recommended to previously dilute the samples in phosphate buffer saline (PBS) (Sluijter et al. 2018).

The main challenges of EVs isolation techniques are to efficiently separate them from other components of the biological sample, such as soluble proteins and lipoproteins, and still obtain a good yield while preserving their original characteristics. However, currently available methods for isolating EVs have advantages and limitations, especially with regard to yield and the presence of contaminants. So far, the main methods used to obtain EVs are differential ultracentrifugation, density gradient ultracentrifugation, size exclusion chromatography (SEC), ultrafiltration, polymer-based precipitation, and immunoaffinity-based techniques (Fig. 2). Two or more isolation techniques can also be combined to obtain the EVs, although it may result in a decrease in the yield. Importantly, the isolation method also directly influences which subpopulation of EVs (e.g., exosomes, microvesicles) is being selected and collected for analysis (Dong et al. 2020).

The choice of techniques for obtaining and studying EVs should be based on their application and downstream analysis while there is still no consensus on the best method for this purpose (Veerman et al. 2021; Azkargorta et al. 2021). Therefore, the method applied for the isolation of EVs is a relevant factor to be considered when comparing studies, analyzing the EV content and, importantly, in the search for biomarkers.

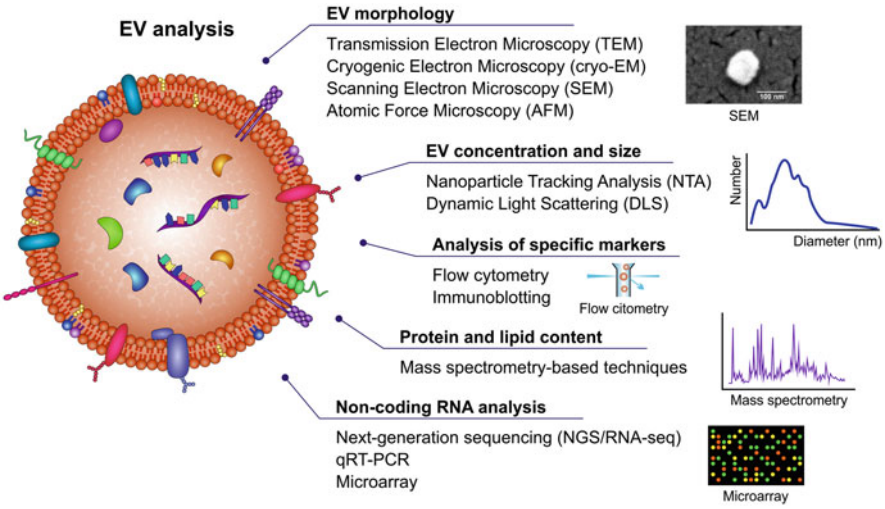

Fig. 2 Main analysis techniques for extracellular vesicles. The analyses performed for the characterization of the EVs are electron microscopy, atomic force microscopy, nanoparticle tracking analysis, dynamic light scattering, flow cytometry, proteomics, transcriptome, lipidomics, and metabolomics

Ultracentrifugation-Based Techniques

Differential ultracentrifugation and density gradient ultracentrifugation are methods that are based on using the force generated by ultracentrifugation to separate the EVs. The differential ultracentrifugation method is widely used and relies on sequential centrifugations that stepwise precipitate larger components and, lastly, the EVs. The procedure usually has centrifugation steps at low (~1,000 x g) and intermediate spin speed (~20,000 x g) to remove cells and cell debris, followed by high-speed ultracentrifugation (~100,000 x g) to precipitate the EVs fraction. However, one of the disadvantages of this method, especially using serum or plasma samples, is the co-precipitation of high molecular weight proteins and protein aggregates. In contrast, density gradient ultracentrifugation separates EVs according to their density. For this, the samples are placed in tubes pre-filled with a solution with a density gradient in a continuous or discontinuous way (in layers), increasing the density from the top to the bottom of the tube. The most used solutions are sucrose, iodixanol, and iohexol. Samples are subjected to high-speed ultracentrifugation and EVs are collected according to density, ranging between 1.1 and 1.19 g/mL. Nonetheless, other components with similar density to EVs, such as high-density lipoproteins (HDL), can be collected together. The disadvantages of using ultracentrifugation techniques are expensive equipment and time-consuming. In addition, standardization of the procedure may be necessary since the centrifugation time, speed, rotor type, and sample viscosity are parameters that may influence the collection of EVs (Kang et al. 2017; Coughlan et al. 2020).

Size Exclusion Chromatography (SEC)

The SEC's principle is to separate EVs according to their size. Briefly, the sample is passed through a column with a porous stationary phase, allowing the larger molecules to pass through more quickly while the smaller ones are withheld in the pores. As a result, the retention time of EVs is different from that of soluble contaminating proteins, which makes it possible to obtain EVs with greater purity. SEC is also a method that provides low mechanical stress to EVs, preserving their primary characteristics (Mol et al. 2017).

Ultrafiltration

Ultrafiltration is based on the selection of EVs by size through a membrane, a process mediated by centrifugation, air pressure, or vacuum pressure force. The membrane used has a cut-off based on the molecular weight that selects EVs, but can also allow contaminating proteins to pass through. Ultrafiltration is often used in combination with centrifugation. However, the filtration process may lead to EV deformation and membrane clogging, which leads to the loss of EVs in the membrane. To overcome this problem, more recently tangential flow filtration (TFF) has been applied to collect the EVs. In this technique, the fluid with the EVs runs tangentially through the membrane, in which molecules smaller than the EVs pass under pressure. The EVs, compounds larger than the cut-off, do not pass the membrane and recirculate the system (Busatto et al. 2018). Through this procedure, TFF enables to concentrate the EVs, which is especially interesting for diluted samples.

Polymer-Based Precipitation

Polymer-based techniques consist of adding a polymer to the samples to reduce the solubility of the EVs, followed by their precipitation by centrifugation at low speed (~1,500 x g). An example of a polymer used is polyethylene glycol (PEG) (Lee et al. 2021). The advantages of this method are the use of conventional centrifuges, being quick to perform, and the preservation of the characteristics of EVs. This technique is also commonly used in several commercial kits. However, the presence of polymers may impair further analyses, such as mass spectrometry. In addition, some studies point to the presence of contaminants by this technique (Azkargorta et al. 2021).

Immunoaffinity-Based Techniques

Immunoaffinity-based techniques isolate EVs through the interaction of their proteins with antibodies or ligands that are immobilized on a solid base, such as a

column, magnetic beads, and others. This interaction allows a subpopulation of EVs to be selected due to high specificity directed to a particular EV protein, separating it from the other components of the sample. Like the previous method, multiple commercial kits are based on immunoaffinity to isolate EVs. This technique can also be coupled with surface plasmon resonance (SPR) biosensors, in which the target EVs present in the sample interact with antibodies immobilized in the biosensor and generate a detectable signal (Choi et al. 2020).

Analysis of Endothelial EVs

The characterization of EVs is an important step in not only determining the results of the isolation method but also establishing biomarkers or functions that are associated with the isolated EVs. Another difficulty is analyzing a specific subpopulation of EVs of interest from biological samples. Like EV isolation techniques, there isn't a single method that fulfills the full spectrum of EV properties, so this step should use multiple methods, considering the experimental question and disadvantages of each approach. The most used methods are transmission electron microscopy (TEM) to analyze the structure of EVs, nanoparticle tracking analysis (NTA) to quantify the number of EVs and their size distribution, flow cytometry, and immunoblotting to identify specific proteins to confirm EV origin and classification (microvesicles or exosomes). In particular focusing on the potential of EVs as biomarkers, other methods based on proteomics, transcriptomics, and lipidomics are also being greatly utilized.

Electron Microscopy

Because of their size, EVs can't be detected by optical microscopes, even the most enhanced ones, thus electron microscopy is applied to analyze their morphology and size. TEM is the most utilized type of electron microscopy for imaging EVs. On TEM, an electron beam passes through an ultra-thin sample, that has been fixed, dehydrated, and later embedded and mounted on a carbon coating grid. The passage of electrons through the sample creates a 2D image of the EVs, showing their inner structure (Chuo et al. 2018; Malenica et al. 2021). Another electron microscopy method used is scanning electron microscopy (SEM) which gives information about EVs topography, including size, shape, and morphology (Malenica et al. 2021). In SEM samples are chemically fixed and dehydrated, then the immobilized samples are coated with a thin layer of conductive material like gold or carbon before imaging. This thin layer of gold and the process of fixation and dehydration may affect the surface structure of EVs, and they can appear saucer-shaped rather than round-shaped (Hartjes et al. 2019; Malenica et al. 2021). Cryogenic electron microscopy (cryo-EM) is also usually chosen to study EVs. In cryo-EM, samples are vitrified at very low temperature (below −100 °C), maintaining their hydrated native state, diminishing the deformation of EV structure by dehydration, and can be used

to determine the ultra-structure of EVs. When combined with immunogold labeling, cryo-EM can image the protein-containing EVs as well as track EV uptake by other cells (Chuo et al. 2018; Hartjes et al. 2019; Malenica et al. 2021).

Atomic Force Microscopy (AFM)

AFM is a nanoscale tool (resolution limit around 1 nm) used to determine the morphology, structure, and composition of nanometric structures. It uses a probe that scans through the surface of samples and a laser beam that measures the probe position changes. Through recording the probe positions, AFM generates topographic images of the samples. AFM can be performed in air mode, where sample preparation requires EVs to be dried and immobilized, or in liquid mode, in which EVs samples are directly measured. The liquid mode has the advantage to maintain the hydrated status of EVs, as well as their native morphology. AFM probes also can be combined with antibodies, allowing quantifying and image EVs with specific surface proteins (Hartjes et al. 2019; Malenica et al. 2021).

Nanoparticle Tracking Analysis (NTA)

The most widely used method to quantify and describe the size distribution of EVs is NTA. This technique is based on how fast a particle diffuses in a static solution due to the Brownian motion. The camera attached to the NTA track and record the motion of each particle, and through a thorough analysis of their trajectories, it's possible to estimate EVs concentration and size distribution. NTA ensures fast detection of EVs and is capable of characterizing particles around 30 nm (Hartjes et al. 2019; Azevedo et al. 2021).

Dynamic Light Scattering (DLS)

DLS is also a technique used to determine the size distribution of vesicles with properties similar to NTA. However, unlike NTA, DLS has limitations in analyzing polydisperse samples. Large particles could interfere with the size determination of smaller particles, or even a group of multiple vesicles can be signed as a single vesicle. Moreover, large protein aggregates could not be differentiated from interested EVs (Pearson et al. 2017; Hartjes et al. 2019; Azevedo et al. 2021).

Flow Cytometry

Flow cytometry is one of the main methods used for the characterization of EVs, although its limitations. EVs are detected by light scattering and fluorescent labeling through specific surface antibodies (Azevedo et al. 2021). In particular, endothelial

EVs can be detected by CD31, CD144, CD146, Annexin V (that binds to phosphatidylserine on the surface of microvesicles), or exosomes surface markers, such as CD9 and/or CD63 (Favretto et al. 2019; Mathiesen et al. 2021; Nederveen et al. 2021). Due to EVs' small size, detection by flow cytometry is still challenging and most conventional flow cytometers are only able to detect EVs above 500 nm. Smaller EVs are detected in clusters, an event called swarm effect in which EVs are simultaneously illuminated by the laser, and their combined scattering rises above the detection limit, resulting in counting them as a single particle. To overcome this, some solutions have been described, like promoting serial dilutions to the sample, bead-based EV assays, or, more recently, using imaging flow cytometers. With the increase of EVs study field, high-sensitivity flow cytometers have also been developed to analyze nanoscale particles (Hartjes et al. 2019; Azevedo et al. 2021).

Proteomics

The protein content of EVs has been greatly studied in recent years, not only to identify and characterize the EVs but also because their protein content can be used as biomarkers. Immunoblotting assays, such as dot blot or western blot, are commonly used to identify EV-associated proteins (CD9, CD63, ALIX, Tsg101) to confirm the presence of EVs in the samples. Immunosorbent assays are also used, in which EVs are captured on a supporting surface coated with antibodies against surface EVs proteins. The subsequent washing steps allow for the elimination of non-associated proteins that could interfere with EV analysis. Immunosorbent assays have the advantage of quantification EV surface proteins without prior EV isolation and/or purification, allowing a faster identification, especially in complex samples such as urine or blood (Hartjes et al. 2019; Nederveen et al. 2021).

Recently has been reported that EVs contain proteins with post-translational modifications (PMTs). Alterations in PMTs in proteins are thought to be determinants in the early progression of diseases, and so have become targets for indication of the cellular states in diseases, especially in cancer and neurodegenerative diseases. Mass spectrometry techniques are used to determine the proteomic content of EVs and PMTs, and although there is no standardized method, multiple mass spectrometry-based protocols have been introduced to analyze the proteomic profile and PMTs of EVs (Andaluz Aguilar et al. 2020; Azevedo et al. 2021).

Transcriptome

RNA represents one of the most important biomolecules associated with EVs, but the specific characterization of RNA in EVs remains challenging and isolation method dependent (Turchinovich et al. 2019). The approaches most used in the transcriptomic analysis of EVs are described below.

qRT-PCR

This method is ideal for when it is necessary to quantify the levels of an RNA or DNA sequence. In the case of RNA analysis, the reverse transcription (RT) product from RNA to cDNA is used in the reaction and then the cDNA amplification is performed using sequence-specific primers optimized for qPCR. The principle of the technique is the detection of the increase of fluorescent signal probes that bind to the nucleic acid as a certain transcript is amplified (Taylor et al. 2010). The biggest advantage of this approach is the small volume of sample required, high sensitivity, and the option to choose relative and/or absolute quantification. Previous studies have relied on qRT-PCR to perform comparative profiles of miRNAs in EVs, such as Chevillet et al. quantified the stoichiometric relationship between the number of EVs and the number of miRNAs per EV (Chevillet et al. 2014). However, with qRT-PCR it is not possible to detect the total amount of RNA, that is, it is only useful when the purpose is to detect and quantify specific sequences.

Next-Generation Sequencing (NGS/RNA-seq)

As one of the most advanced methods for reading nucleic acid sequences, this approach requires three steps (Gandham et al. 2020): (I) library preparation using PCR or cDNA product, DNA fragmentation and linkage to adapters, and hybridization in the sequencer, which is covered with complementary oligo sequences for further bridging amplification; (II) sequencing through fluorophore incorporation and nucleotide synthesis; and (III) data processing, which involves alignment with a reference sequence.

The feasibility and applications of sequencing small RNAs in EVs have been reported (Huang et al. 2013; Amorim et al. 2017). The advantage of RNA-seq lies in its ability to identify and profile RNA subtypes in various EV subpopulations. A recent study conducted to characterize extracellular RNA (exRNA) released from human glioma stem cells using NGS found that the RNA profiles of microvesicles, exosomes, and ribonucleoproteins differ substantially (Wei et al. 2017).

Both RNA-seq and microarrays are used to achieve clinical end-point, but RNA-seq is more efficient in detecting low-abundant transcripts, distinguishing isoforms, and allowing the identification of genetic variants (Zhao et al. 2014). However, this approach has biases, such as the selection of methods, kits, and platforms for RNA isolation, library preparation, ligation, and sequencing type (e.g., HiSeq or MiSeq, SOliD, Ion Torrent), as well as bioinformatics parameters in the processing of data in normalization methods (Mateescu et al. 2017). In addition, the method is time-consuming and expensive (Gandham et al. 2020).

Microarrays

Microarrays are often used to study the global profiles of hundreds of genes in biomedical samples and allow for the differential analysis of RNA/DNA samples. This technique is based on DNA probes that are deposited on a chip in an "array" format and bind to complementary target gene sequences in the samples (Murphy 2002; Bumgarner 2013).

In a recent study performed on EV-RNA from mast cells using the microarray data combined with NGS, four different clusters of two distinct exRNA tags [high-density (HD) and low-density (LD) exRNA] were identified (Jaksik et al. 2015). The high throughput and simultaneous measurement of thousands of mRNA transcripts for gene expression or genomic DNA fragment to allow the analysis of copy number variation are among the main advantages of microarrays (Gandham et al. 2020). Regardless, a secondary confirmation step, such as qRT-PCR, is usually required to verify some of the key genes, depending on the purpose and scope of the experiment (Jaluria et al. 2007). Disadvantages of microarrays include high cost, access to specialized equipment, and numerous probe designs based on low specificity sequences, as well as dependence on selected transcript pools for analysis of the designed probe sets (Jaksik et al. 2015).

Other Techniques

The content of EVs is also formed by lipids and metabolites. Although small in number, they can be transferred to recipient cells and induce a response. Lipids like arachidonic acid was shown to activate cyclooxygenase 2 (COX2) and prostacyclin synthesis (Barry et al. 1997; Hartjes et al. 2019; Desideri et al. 2021; Nederveen et al. 2021; Azevedo et al. 2021). Studying the metabolic and lipidic content of EVs is important to understand biological mechanisms, their role in pathological conditions, and also use these molecules as potential biomarkers. Mass spectrometry-based platforms and thin-layer chromatography (TLC) assays are being used to analyze the lipidic content, and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) and gas chromatography-mass spectrometry (GC-MS), along with specific protocols to metabolic extraction, are the chosen to study the metabolic content (Luo et al. 2018; Williams et al. 2019; Chen et al. 2019; Dudzik et al. 2021).

Endothelial EVs in Cardiovascular Diseases and Kidney Diseases

Endothelial cell-derived EVs are closely related to diseases that induce endothelial dysfunction, including CVDs and CKD that are deeply connected, ultimately leading to the cardiorenal syndrome. So far, studies have focused on analyzing the number of EVs formed under these pathological conditions and their protein and miRNA content. However, many of these studies evaluated the content of total circulating EVs, without specifying the subpopulation or cellular origin. It is worth noting, therefore, that the analysis of circulating EVs in patients with CVD and kidney diseases is recent and further studies are needed, especially regarding endothelial EVs and the interconnection between the cardiovascular and renal systems.

EVs and Cardiovascular Diseases

CVDs are strongly related to endothelial activation and dysfunction, which may also affect the formation of EVs. An increase in the amount of endothelial EVs is usually seen in blood samples from patients with CVD, including coronary artery disease (CAD) and heart failure (HF) (Bernal-Mizrachi et al. 2003; Biasucci et al. 2012). Even after months of an acute coronary syndrome (ACS) event, endothelial and platelet EVs with pro-thrombogenicity properties remain at high levels in the blood, reflecting the state of endothelial injury and inflammation (Koganti et al. 2021). In a clinical study, it was reported that patients with ACS (N = 64) had higher levels of endothelial EVs than patients with stable angina (N = 20), both being significantly higher than the control group (N = 42) (Bernal-Mizrachi et al. 2003). This effect can be verified not only in the ACS but also in the therapeutic intervention that follows it. An increase in the number of circulating endothelial and platelet EVs was observed in patients with ACS (N = 43) after 1 day of percutaneous coronary interventions, with a subsequent decrease on the second day; however, no difference was observed in patients with stable angina (N = 33) over the 2 days analyzed (Biasucci et al. 2012). Increased levels of endothelial EVs is also a predictor of future cardiovascular outcomes in patients at high risk for coronary heart disease (Nozaki et al. 2009).

Circulating endothelial EV levels could be also enhanced in patients with risk factors for CVD, such as hypertension and diabetes (Tramontano et al. 2010; Sansone et al. 2018). Considering the specific type of endothelial EVs, it was recently reported that the ratio of CD62E+/CD31+ EV subpopulations is reduced in patients with type II diabetes mellitus (N = 20) and even more in patients with diabetes and ACS (N = 20) compared to the control group (N = 20), which suggests a greater presence of endothelial cells apoptosis under these pathological conditions (Marei et al. 2022).

Differences in the protein content of circulating EVs from patients with CVD are also observed. The proteomics analysis conducted by Cheow et al. (2016) reported increased levels of 252 proteins in circulating EVs from patients after myocardial infarction (N = 15) compared with patients with stable angina (N = 20) (Cheow et al. 2016). In another clinical study, Gidlöf et al. (2019) demonstrated a reduction in chemokine ligand 17 (CCL17), chymotrypsin C (CTRC), and proto-oncogene tyrosine-protein kinase SRC (SRC) levels in the EVs from patients with myocardial infarction (N = 60) compared to a healthy group (N = 22), which was not observed in the analysis of total plasma. The levels of EVs with SRC were lower in patients with ST-elevation myocardial infarction (STEMI) compared with those with stable angina pectoris, making it possible to verify differences between groups of CAD. However, the process involved in the reduction of SRC and in which the EV subpopulation is still unknown (Gidlöf et al. 2019).

EVs likely play an effector role in the plaque formation in atherosclerosis, which is the main cause of CAD. In vitro, endothelial cells exposed to EVs isolated from atherosclerotic plaques had an increase in intercellular adhesion molecule 1 (ICAM-

1) levels as well as in monocyte adhesion, which demonstrates that EVs may be contributing to the pro-inflammatory state of the endothelium in atherogenesis (Rautou et al. 2011). An increase in the expression of pro-inflammatory molecules (i.e., IL-1β, TNF-α, and ICAM-1) is also observed in endothelial cells exposed to circulating EVs isolated from the serum of patients with CAD (Zhang et al. 2021).

miRNAs are another important molecule that EVs carry. Due to their incorporation into extracellular vesicles, miRNAs have high stability in fluids and are detectable in the blood (Weber et al. 2010). Many studies have shown that the expression levels of circulating miRNAs in both tissue and plasma are altered in some cardiovascular diseases. Thus, circulating miRNAs have recently emerged as a new class of biomarkers in cardiac patients (Loyer et al. 2014). Studies have tried to propose that the transfer of EVs associated with miRNAs can modulate the biological functions of the acceptor cell (Jansen et al. 2013). The high expression levels of vasculoprotective miRNAs within EVs have an atheroprotective effects in experimental murine models with vascular injury and atherosclerosis, being associated with a reduction in adverse cardiovascular events in patients with stable CAD (Jansen et al. 2013, 2014). Uptake of miR-143/145 containing EVs is also related to atheroprotection in smooth muscle cells (Hu et al. 2019). However, it is not yet known whether vascular or myocardial miRNAs are selectively packaged into circulating EVs in response to atherosclerotic conditions (Liu et al. 2019).

Furthermore, the relationship between miRNA expression levels and incidents of myocardial infarction was observed by Zampetaki et al. (2012), who found significant differences in the levels of three miRNAs (miR-126-3p was positively associated, while miR-197 and miR-223 were inversely associated) (Zampetaki et al. 2012). MiR-126-3p derived from endothelial EVs modulates vascular smooth muscle cell proliferation and stops neointimal formation by inhibiting the secretion of low-density lipoprotein receptor-related protein 6 (Jansen et al. 2017). Given the importance and differences of miRNAs in pathological conditions, the specific signatures present in miRNA expression have potential use as diagnostic and prognostic tools (Vavassori et al. 2022).

EVs and Kidney Diseases

Endothelial EVs appear to play a role in endothelial dysfunction in kidney diseases, especially CKD. Some clinical studies have shown that there is an increase in endothelial EVs in patients with pre-dialysis CKD and also in those on hemodialysis or peritoneal dialysis (Faure et al. 2006; Merino et al. 2010). Pediatric patients with pre-dialysis CKD had higher levels of endothelial EVs than those patients on dialysis, and both were higher than the healthy group (Dursun et al. 2009). On the other hand, it was demonstrated in a randomized trial that patients with stage 3 and 4 CKD treated with paricalcitol (active vitamin D) had a reduction in the levels of ICAM-1 positive endothelial EVs, which the authors suggest is an indicator of a less pro-atherosclerotic endothelium due to the vasoprotective role of vitamin D (Lundwall et al. 2019).

The increased formation of endothelial EVs may be related, at least in part, to exposure to uremic toxins, which are compounds that accumulate in the body with loss of kidney function. For example, it has been shown in vitro that the uremic toxin *p*-cresyl sulfate induced the release of EVs by endothelial cells (Meijers et al. 2009; Favretto et al. 2021). EVs generated in the uremic environment may have a modulating role in the vessel, although their effects are not yet fully understood. Recently, Favretto et al. (2021) demonstrated in vitro that endothelial EVs derived from cells exposed to *p*-cresyl sulfate, indoxyl sulfate, and inorganic phosphate had varied effects on the expression of pro-inflammatory molecules (VCAM1), cell migration and adhesion (Favretto et al. 2021).

Uremic toxins also contribute to the formation of endothelial EVs with procoagulant properties. In vitro, EVs derived from endothelial cells exposed to indoxyl sulfate and indole-3-acetic acid showed higher production of factor Xa due to tissue factor activity, whose expression is also increased in the source cell of EVs (Gondouin et al. 2013). Endothelial EVs formed under conditions of hyperphosphatemia also induce thrombin formation (Abbasian et al. 2015). The same effect is observed in EVs isolated from patients with CKD. Burton et al. (2013) reported that patients with CKD on hemodialysis (N = 20) or peritoneal dialysis (N = 17) have higher levels of EVs with procoagulant properties than the healthy group (N = 20) (Burton et al. 2013).

Proteomic analysis demonstrated that patients with CKD stages 3 and 4 have circulating EVs with higher levels of pro-inflammatory proteins, including complement factor D and serine protease (PRSS2), compared to the healthy group (Jalal et al. 2021). The presence of these proteins reflects the pro-inflammatory state of the endothelium. Interestingly, the same study also found higher levels of β2-microglobulin and cystatin C, both protein uremic toxins, in circulating EVs from patients with CKD (Jalal et al. 2021).

EVs carry miRNAs that play an important role in vascular homeostasis, but their levels may be altered in CKD. The study conducted by Shang et al. (2017) identified higher miR-92a levels in endothelial EVs from patients with CKD and also in rats with adenine-induced renal dysfunction (Shang et al. 2017). This miRNA is known for its role in angiogenesis and atherogenesis, contributing to the development of CVD. In another study, lower levels of miRNAs with a vasoprotective role (miR-126-p and miR-130a-3p) were found in circulating EVs from patients with CKD and CAD and also in EVs derived from endothelial cells exposed to uremic toxin indoxyl sulfate (Zietzer et al. 2020). Studies are constantly providing new data on miRNAs, and it is challenging to turn these experimental findings into reliable clinical diagnostic tools (Zang et al. 2019; Franczyk et al. 2022). Studies indicate that some miRNA levels may be increased in patients with CKD stage III–V and on hemodialysis and decreased in kidney transplant recipients (miR-143, miR-145, and miR-223) (Cordes et al. 2009; Metzinger-Le Meuth and Metzinger 2019; Fourdinier et al. 2019), as well as elevated in patients with CKD stages III–V, decreased in hemodialysis patients, and even more decreased in kidney transplant recipients (miR-126 and miR-155) (Chen et al. 2013; Fourdinier et al. 2019).

Studies have shown that miRNA from circulating EVs and also from EVs isolated from the urine of patients can be biomarkers of kidney diseases, such as diabetic kidney disease, acute kidney injury, and hypertensive nephropathy (Sun et al. 2016; Nandakumar et al. 2017; Assmann et al. 2019). Since the exosomes present in the urine originate from cells in the various segments of nephrons (Mathivanan et al. 2010), altered miRNA profiles may indicate loss of kidney function (Zang et al. 2019). Recently, two urinary exosomal miRNAs, miR-342-3p and miR-192-5p, were identified to be differentially regulated in patients with renal disease derived from type 2 diabetes mellitus (Assmann et al. 2018). Furthermore, another study implicated three urinary miRNAs (miR-126-3p, miR-155-5p, and miR-29b-3p) as potential biomarkers of this disease (Beltrami et al. 2018). The expression profiles of miR-21-5p and miR-30b-5p are also shown to be altered as a consequence of renal dysfunction, since the increase in urinary exosomal expression of miR-21-5p and the decrease in miR-30b-5p are collectively capable of differentiating individuals with impaired renal function (Zang et al. 2019).

miR-146a has been implicated in the regulation of the immune and inflammatory response (Franczyk et al. 2022). This miRNA was found to be correlated with the infiltration of inflammatory cells during the development of chronic kidney inflammation (Ichii et al. 2012). miR-146a has been linked to the regulation of maturation and survival of human dendritic cells (Giahi et al. 2012; Karrich et al. 2013) and was also shown to be upregulated by the production of inflammatory response cytokines (de la Guardia et al. 2013). Wu et al. (2017) suggest that bone marrow-derived mesenchymal stem cell microvesicles are responsible for miR-146a expression and IL-12 production, which have expanded to suppress dendritic cell maturity, thus improving allograft survival in a model of kidney transplantation in mice (Wu et al. 2017). Therefore, miR-146a in plasma has been considered a biomarker for acute organ transplant rejection (Hu et al. 2013).

A summary of the studies that analyzed the EV content in CVD and CKD is presented in Fig. 3.

Final Considerations

Studies that address the circulating EVs, and more specifically endothelial EVs, in pathological conditions are recent. Despite the promising data with EVs, further studies are still needed to validate the findings with larger patient cohorts and longitudinal approaches. Changes in the EV content in pathological conditions, such as CVD and CKD, could reveal potential disease biomarkers, in which the processes of EV isolation and analysis are meaningful since abundant soluble proteins present in biological samples may mask them. However, the standardization of techniques for isolating and analyzing EVs, as well as their wide availability, are still challenges in the EV investigation. The ability of EVs to transfer biomolecules to recipient cells makes them also a potential tool for therapeutic interventions; however, their application in clinical practice remains to be seen.

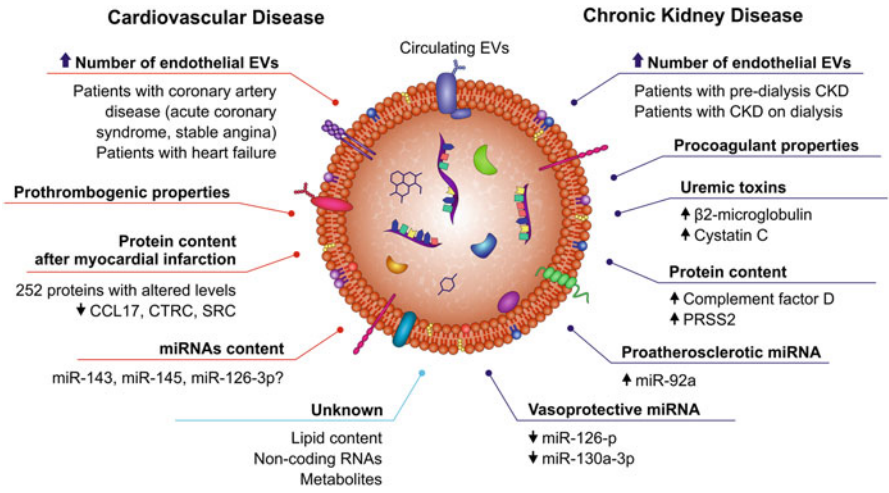

Fig. 3 Characterization of circulating extracellular vesicles in cardiovascular diseases and chronic kidney disease. Studies have shown an increase in the amount of endothelial EVs in cardiovascular disease and chronic kidney disease. The protein and miRNA content of circulating EVs is also altered. However, the lipid content, other non-coding RNAs, and the metabolic profile are still unknown in these pathological conditions

Applications to Prognosis

Endothelial EVs carry bioactive molecules that can contribute to vascular dysfunction, such as miRNA-92a which is linked to the development of atherosclerosis. Shang et al. (2017) demonstrated not only that circulating levels of miRNA-92a are enhanced with CKD progression but also that this increase is present in endothelial EVs (Shang et al. 2017). Interestingly, Wiese et al. (2019) demonstrated that inhibition of miRNA-92a in an animal model with kidney injury led to a reduction in atherosclerotic lesions (Wiese et al. 2019). It is possible that miRNA-92a from endothelial EVs is a biomarker of the proatherosclerotic state of the endothelium in CKD and also in CVD.

Mini-Dictionary of Terms

- Brownian motion: random motion of particles that are suspended in a liquid or gas.
- miRNA: small non-coding RNA molecules that regulate gene expression at the post-transcriptional level by binding to the 3′ untranslated region (UTR) of target mRNA sequences, promoting mRNA degradation or blocking its translation.
- Percutaneous coronary intervention: a non-surgical procedure performed to open the lumen of the artery with a catheter used to place a stent in the blocked site.

- Post-translational modifications (PMTs): alterations, such as phosphorylation and glycosylation, that can alter protein stability, activity, conformation, and even interaction with other cellular molecules.
- Surface plasmon resonance (SPR): electromagnetic response resulting from the plasmonic field generated from the oscillatory motion of electrons excited by light incident on metal material.

Key Facts of Cardiovascular Diseases

CVD is the leading cause of death worldwide, accounting for 19.2 million deaths in 2019 alone (32% of total deaths).

Heart dysfunction leads to impaired kidney function and vice versa, a condition known as cardiorenal syndrome.

CAD is a subset of diseases characterized by the total or partial blockage of arteries that supply the heart, impairing blood availability. The main cause is the formation of atherosclerotic plaques.

Atherosclerosis is a pathological process intrinsically related to endothelial dysfunction and inflammation in the vessel wall.

Type 2 cardiorenal syndrome corresponds to chronic cardiac dysfunction that results in the development of CKD.

Key Facts of Chronic Kidney Disease

CKD is divided into stages 1 to 5, in which stage 5 corresponds to the patient with renal failure (with an estimated glomerular filtration rate of <15 mL/min/1.73 m^2) and requires the use of renal replacement therapy.

Renal replacement therapies are hemodialysis, peritoneal dialysis, and kidney transplantation.

As CKD progresses, uremic toxins accumulate in the body since dialysis therapies only partially remove them, specially protein-bound uremic toxins, such as indoxyl sulfate and *p*-cresyl sulfate.

CKD leads to impairment of the cardiovascular system, known as type IV cardiorenal syndrome or chronic reno-cardiac disease.

CKD affects multiple biological systems of the body and decreases the patient's life expectancy, with CVD being the main cause of death.

Summary Points

- Endothelial EVs are released by endothelial cells after activation, stress, or apoptosis. They are now to further endothelial cells, inducing responses that lead to inflammation, oxidative stress, and apoptosis.

- Circulating EVs, including those derived from the endothelium, are obtained from serum or plasma and are of interest for use in liquid biopsies.
- The main methods of isolation of EVs are differential ultracentrifugation, density gradient ultracentrifugation, SEC, ultrafiltration, polymer-based precipitation, and immunoaffinity-based techniques. Each technique has advantages and disadvantages to use.
- EVs can be physically characterized by electron microscopy, NTA, or DLS. Their surface proteins can be tracked by flow cytometer, immunoblotting, or immunosorbent assays. The transcriptome can be done by qRT-PCR, NGS/RNA-seq, and microarray. Proteomics methods can be used to analyze the protein content of EVs, which can be altered to induce a response in recipient cells.
- The amount of endothelial EVs is increased in CVD and CKD, which may be related to endothelial dysfunction in these pathological conditions.
- Proteomic and miRNA expression analyses showed changes in the content of circulating EVs from patients with CVD and also patients with CKD.

Cross-References

▶ Extracellular MicroRNAs as Putative Biomarkers of Air Pollution Exposure

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Part IV

Functional and Physiological Variables and Platforms

Biomarkers of Lead Exposure: Platforms and Analysis

23

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Abstract

In this chapter, the main lead exposure biomarkers reported in the scientific literature are summarized, along with their pros and cons and situations in which they should be recommended. The population is exposed daily to lead concentrations present in the environment. Pregnant women and children are the groups most affected by lead exposure and may suffer a number of health effects. Lead exposure surveillance is crucial both for environmental and occupational exposure. Knowing how to select the most suitable biomarker for each exposure scenario is essential to allow useful and effective biomonitoring. Different types of biological matrices can be used to assess biomarkers of lead exposure, including teeth, bone, nails, whole blood, plasma, urine, saliva, and hair. Blood Pb remains the biomarker of choice for recent lead exposure, while long-term exposure can be determined using the mean of serial blood Pb levels. Pb in teeth, and bone can be good biomarkers for investigating cumulative exposure. Pb in urine and saliva reflects recent exposure and has the advantage of being a noninvasive method. However, some factors which vary greatly among individuals need to be taken into account. Nails can serve as a good tool for initial screening.

Keywords

Lead · Exposure · Environment · Occupational · Biomonitoring · Teeth · Bone · Nails · Blood · Plasma · Urine · Saliva · Hair

Abbreviations

ALA	Aminolevulinic acid
ALAD	Aminolevulinic acid dehydratase
BLRV	Blood lead reference values
CDC	Centers for Disease Control and Prevention
EDTA	Ethylenediaminetetraacetic acid
ET AAS	Electrothermal atomic absorption spectrometry
GABA	γ-Aminobutyric acid
GF AAS	Graphite furnace atomic absorption spectrometry
HCl	Hydrochloric acid
HNO_3	Nitric acid
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
ICP-MS	Inductively coupled plasma mass spectrometry
IAEA	International atomic energy agency
LEPAC	Lead Exposure and Prevention Advisory Committee
NHANES	National Health and Nutrition Examination Survey
Na_2EDTA	Disodium ethylenediaminetetraacetic dihydrate
NH_4OH	Ammonium hydroxide
Pb	Lead

SDGs	Sustainable development goals
TMAH	Tetramethylammonium hydroxide
UNICEF	The United Nations Children's Fund
XRF	X-ray fluorescence

Introduction

The population is exposed daily to low lead levels present in the environment and also to high concentrations in lead hot spots or during occupational activities, particularly informal activities, which often lack the proper occupational hygiene (Fig. 1). Pregnant women and children are the groups most affected by exposure to chemical contaminants and may suffer a number of health effects, including cognitive and antisocial behavior in children and adolescents. The United Nations Children's Fund (UNICEF) released the 2020 report, "The Toxic Truth: Children's Exposure to Lead Undermines a Generation of Potential Future," reporting that lead exposure spans the 17 sustainable development goals (SDGs), with 1 in 3 children in the world (approximately 800 million) presenting blood lead levels above the reference values accepted by the World Health Organization (WHO) and the US Centers for Disease Control and Prevention (CDC), calling for urgent global actions to combat this public health problem.

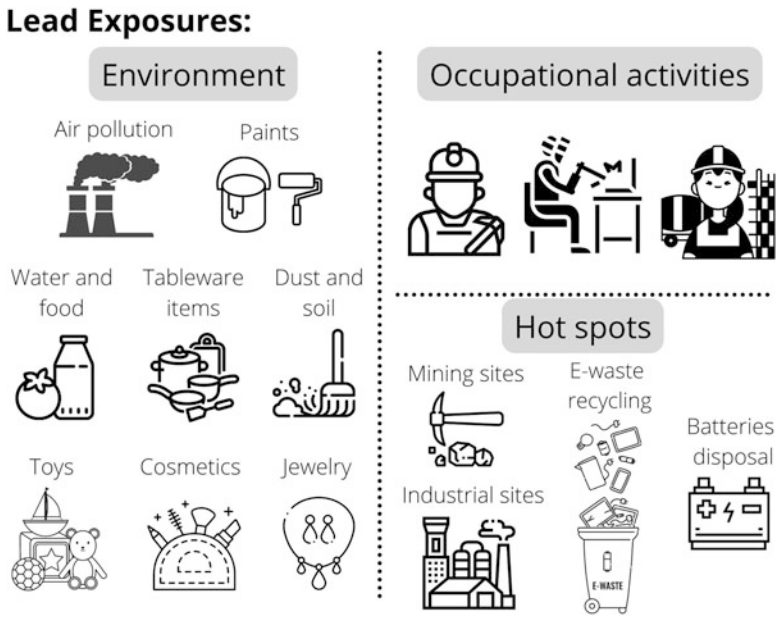

Fig. 1 Different lead exposure sources.
This figure shows different types of lead exposure to which humans can be exposed

Inorganic lead is widely distributed in the environment. The entry of lead into the human body can occur via ingestion and/or inhalation. More specifically, in occupational exposures, inhalation can occur through fumes from soldering lead alloys or metals. After entering the body, lead is distributed through the blood and binds to the bones and soft tissue. The Pb^{2+} ion has an affinity for proteins, compromising the functioning of structures containing them. These characteristics can affect hemoglobin production and cause damage to the central and peripheral nervous systems, nephropathy and vascular problems, etc. (Hoet 2005; Olympio et al. 2009, 2010c).

Given these deleterious effects, lead exposure surveillance is crucial both for environmental and occupational exposure (Olympio et al. 2017), and knowing how to select the most suitable biomarker for each exposure scenario is essential to allow useful and effective biomonitoring. The primary aim (e.g., (1) to investigate recent or remote exposure, (2) to study general population or worker exposure, or (3) to include children or adults) dictates whether the biomarker of choice will yield the best evidence.

Biological Matrices and Lead Exposure Biomarkers

Different biological matrices can be used to assess biomarkers of lead exposure (Fig. 2). In this chapter, each of these biomarkers will be discussed in detail.

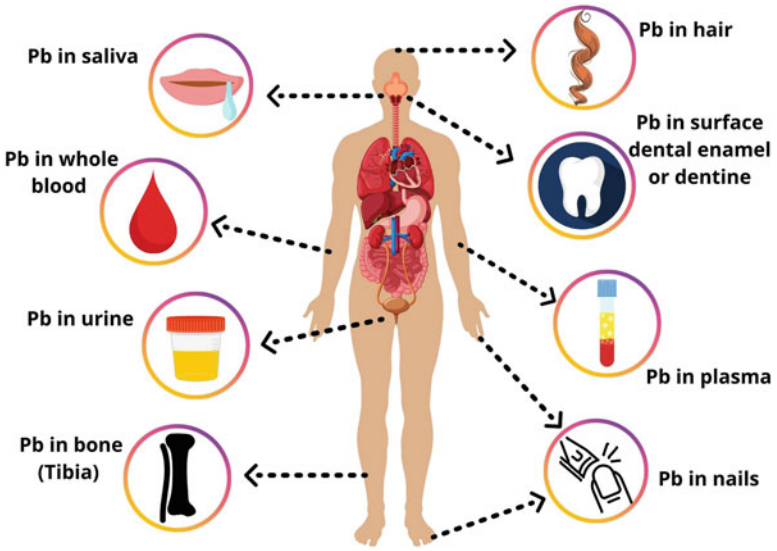

Fig. 2 Biomarkers of lead exposure.
This figure shows all the biomarkers of lead exposure covered in this chapter

Teeth

Remote exposure biomarkers can be used to evaluate remote or chronic lead exposure, for example, to assess whether adolescents with antisocial behavior were exposed to Pb during central nervous system development (Olympio et al. 2009). Teeth lead concentrations are widely recognized biological markers of childhood lead exposure because deciduous teeth accumulate lead during their embryonic and postnatal life (NRC 1993).

Dentine lead can be used to assess exposure to different environmental risk factors for lead exposure. Dentine lead levels can be used to investigate the long-term effects of early lead exposure, including during childhood and prenatal periods (Arora et al. 2014). Dentine is characterized by three different types: (a) primary dentine, which forms before tooth eruption; (b) secondary dentine, which develops after eruption from the odontoblasts living within the pulp and is laid down in layers within the pulp cavity; and (c) reparative or tertiary dentine, which results from thermal, chemical, bacterial, or mechanical trauma to the odontoblasts. This type is darker in color and dense in structure and has few tubules. According to Arora et al. (2014), measurements of Pb in dentine can be reliably used to reconstruct Pb past exposure history but also have the potential to estimate timing of exposure up to the moment the tooth is shed, allowing the investigation of critical windows of susceptibility. Pb levels in dentine at birth were significantly associated with umbilical cord blood Pb, while prenatal dentine Pb was associated with maternal patella Pb. In assessments of postnatal exposure, Pb levels in 3-month dentine were significantly associated with Pb concentrations in children's blood, and mean Pb concentrations in secondary dentine correlated with cumulative blood lead.

Dentine lead determination is usually based on the collection of naturally shed deciduous teeth. Each child should supply at least one tooth (Fergusson et al. 2008; Arora et al. 2014), which can be stored dry at room temperature. Information on the reason the tooth was shed and age at shedding are important (Arora et al. 2014).

Another way of using teeth to measure lead exposure is by evaluating surface dental enamel lead levels through in vivo microbiopsies (Gomes et al. 2004; Olympio et al. 2010a, b, c, 2018a). Lead determination in surface dental enamel is a reliable way of measuring remote exposures to Pb in a rapid, safe, and painless procedure (Olympio et al. 2018a). Biopsy depths depend on the pH of the extraction solution and on its residence time on the surface dental enamel. Exposure to acid for a short period removes a shallower layer of dental enamel. The measurements of lead concentrations in tooth enamel using chemical and physical methods for different biopsy depths converge to values similar to those made using the conventional cylinder approach (Olympio et al. 2018a). This information is crucial, as deeper biopsies contain less lead than superficial ones, since surface enamel has a steep lead gradient (Brudevold et al. 1977). Notably, the surface dental enamel of permanent teeth that have erupted is not susceptible to environmental lead intake, making it a good biomarker of remote lead exposure. A statistically significant association between lead exposure and antisocial behavior in adolescents has been confirmed by longitudinal and cross-sectional studies, irrespective of the biological sample

(blood, bone, or tooth enamel) used (Dietrich et al. 2001; Needleman et al. 2002; Wright et al. 2008, 2009; Olympio et al. 2009, 2010a, b, c; Arbuckle et al. 2016) or the cultural environment of the population cohorts investigated. However, given the lack of reference values for lead in teeth, levels can only be compared against the same study groups to which the same methodology has been applied.

For collecting surface dental enamel samples, teeth need to be cleansed and dried, and an adhesive tape placed firmly onto the labial surface of the subject to delimit the biopsy site. This biopsy procedure has been described by Olympio et al. (2010c, 2018b) and Gomes et al. (2004).

Bone

Bone lead, usually tibial, can also be used to evaluate cumulative exposure for chronic lead absorption. Epidemiologic studies have used bone lead levels as a biomarker of lead exposure in environmental exposure populations, such as children with attention-deficit hyperactivity disorder (Needleman et al. 1996, 2002; Lin et al. 2019) or the older population (Specht et al. 2018). Bone lead can be an important source of steady-state blood lead concentration, even considering environmental exposure. Lead can accumulate to produce a skeletal burden that remains the predominant source of blood lead concentration even after exposure ceases. Therefore, previous exposures may contribute to total blood lead concentration, especially during pregnancy and senescence, reflecting the long half-life of removal from the bloodstream (Vigeh et al. 2011; NRC 1993). During periods of bone activity or demineralization, such as pregnancy, lactation, or osteoporosis events, mobilization of Pb stored in the bones into circulating blood occurs (Hu et al. 1991; Shahida et al. 2021). Furthermore, lead exposure may be a risk factor for the development of osteoporosis, since Pb may replace Ca from the bones and affect osteoblast function (Shahida et al. 2021).

Bone lead levels can also be evaluated in occupationally exposed populations, especially in cases of prolonged exposure (Hu et al. 1991; Landrigan 1991; Vigeh et al. 2011; Barry et al. 2019). Bone Pb measurements are normally performed using X-ray fluorescence equipment, and the measurement site is the middle of the tibia. A K-shell X-ray fluorescence (KXRF) device is the most commonly used, and each analysis takes about 30 min (Specht et al. 2018; Barry et al. 2019; Lin et al. 2019). Before measurement, the subject's legs are cleaned using alcohol and ethylenediaminetetraacetic acid (EDTA) to remove any Pb contamination. The study participant is placed in a sitting position with their right leg immobilized during the analysis (Hu et al. 1991; Landrigan 1991; Specht et al. 2018; Barry et al. 2019; Lin et al. 2019).

Nail

Nail lead levels are a biomarker suitable for investigating subchronic environmental lead exposure. The collection of nail samples is noninvasive, and the samples are easily transported and stored, making the process economically viable. Disclosing

exogenous exposure, nail tests constitute a good tool for initial screening (Oliveira et al. 2021). A toenail can represent an exposure period of 2–12 months, versus 5–6 months for fingernails (Ab Razak et al. 2015; Sakamoto et al. 2015). Fingernail lead levels seem to serve as an indicator of lead exposure sources in contact with the individual, but not as a reliable biomarker of internal dose (Olympio et al. 2020; Oliveira et al. 2021). External contamination is a major concern in the pretreatment of nail samples. Therefore, comparison of the results of different studies is hampered by the heterogeneity of methods of analysis and preparation (Błażewicz et al. 2017). Oliveira et al. (2021) concluded that lead levels in nails did not show a significant difference for a range of exposure levels to the element, as measured in the blood. Only weak correlations between nail and blood lead levels were detected. Although the correlation between mean nail lead levels and the appearance of nails was not statistically significant, nails that were abnormal in appearance contained higher nail lead levels, suggesting external contamination, even after washing of samples.

For the collection procedure, research participants should ideally refrain from cutting their nails for 15 days to ensure a sufficient sample of nail clippings (Oliveira et al. 2021). Clippings measuring at least 1 mm in height should be collected from the thumbnails of both the left and right hands using a pair of stainless-steel nail clippers. Nail polish should be removed using acetone (Sakamoto et al. 2015). After collection, the samples can be stored in ribonuclease (RNase)-free microfuge tubes, decontaminated for trace elements and then kept at room temperature until sample preparation and analysis (Olympio et al. 2020; Oliveira et al. 2021).

Blood

Blood lead of exposed populations remains the biomarker of choice for lead exposure (Barbosa Jr et al. 2005; Olympio et al. 2018a), when the aim is recent exposure evaluation. Blood lead levels generally reflect recent exposure that has occurred 20–30 days before measurement. However, long-term Pb exposure can be assessed by measuring the mean of serial blood Pb levels over longer periods, providing a better assessment of temporal fluctuations in Pb absorption (Barbosa Jr et al. 2005). Blood lead concentrations can be assessed in children and adults to investigate possible sources of contamination or for environmental monitoring in population-based studies. Blood lead levels may aid investigation of associated risk factors in environmentally exposed children and adults. In this case, blood lead can be evaluated in conjunction with the exposure sources, albeit the diet, air, dust, soil, or home and school environments (Leroux et al. 2018; Olympio et al. 2018a).

In occupationally exposed populations, blood lead as a biomarker is an important tool to assess the level of workers' recent exposure (Mohammadyan et al. 2019; Ferreira et al. 2019). Occupational exposures are often responsible for high blood lead levels reported among adults. Therefore, the use of this biomarker may identify risks of lead poisoning and investigate health risks related to this exposure (Rinsky et al. 2018; Ferreira et al. 2019).

The collection of whole blood is the best practice to measure lead exposure. Initial screening capillary tests can be used but need confirmatory testing using a venous sample (CDC 2021). Blood should be drawn from the patient's arm by a certified phlebotomist. The Centers for Disease Control and Prevention recommendation (CDC 2018) is to invert the tube eight to ten times after drawing the blood sample to prevent clotting and ensure good distribution of the additives throughout the blood sample. Vials should be duly labeled after collection. No fasting or special diets are required before blood collection for Pb determination. Contamination of blood samples during the collection process by the external environment is a major concern because lead is ubiquitous. The main precautions are cleaning the collection area and covering the surface with a sterile disposable pad, using prescreened or known lead-free supplies, including sterile collection devices labeled "metal-free," "for trace elements," or "for lead testing," because only the "sterile" designation does not indicate the device is free of contamination by metals. If possible, include one or more field blanks (empty collection tubes) to evaluate potential metal contamination in the analytical phase. If the study involves the collection of blood for other analyses, always take the lead sample first to avoid contamination (CDC 2018).

Plasma

Plasma Pb levels reflect the fraction of circulatory Pb that is more freely available for exchange with tissues. For this reason, some studies consider it a relevant index of exposure and health risks associated with Pb (Barbosa Jr et al. 2005). Evidence suggests that plasma and blood Pb ratios vary between and within individuals, where this disparity may be attributable to underlying differences in toxicokinetics (Smith et al. 2002). According to Sommar et al. (2014), plasma lead testing should be considered for exposed individuals, but not for low exposures in the general population. The lack of reference values hinders comparisons of plasma lead levels.

Plasma collection should follow the same procedures and precautions described earlier for blood. Blood samples should be collected by venipuncture into a tube containing anticoagulants for plasma separation. After collection, the sample should be centrifuged at room temperature. The plasma fraction needs to then be transferred to a metal-free microtube or polyethylene tube and immediately frozen (Smith et al. 2002; Barbosa Jr et al. 2006). After blood collection, plasma separation must be performed as soon as possible because there is high potential for hemolysis, during which Pb moves from erythrocytes into the plasma, leading to erroneously high results for plasma Pb (Smith et al. 2002).

Urine

Lead in urine reflects recent Pb absorption and is the most widely accepted biomarker for trace metal exposure after blood (Esteban and Castaño 2009; Gil et al. 2011; Molina-Villalba et al. 2015). Urinary lead levels depend on the elimination of

Pb by the kidney, which can fluctuate and may not rise significantly following chronic Pb poisoning (Nouioui et al. 2019). Variations in the volume of urine produced both within and between individuals may result in substantial variations in lead concentration, and creatinine correction has therefore been used to adjust for variations in hydration status (Aylward et al. 2014; Salles et al. 2021). Compared to other matrices, urine is more accessible and available in large volumes. In addition, urine collection is a low-cost, noninvasive method that can be applied to all participants easily. Also, the technique allows low lead concentration determinations (Zhang et al. 2017; Salles et al. 2021).

Ideally, 24-hour urine collection should be performed, but this is often logistically difficult. Hence, participants are instructed to provide the first urine in the morning by collecting only the normal urine flow and not the initial discharge (CDC 2018; Zhang et al. 2017; Salles et al. 2021). Externally threaded containers are preferred to minimize contamination of the specimen and prevent leaks. Lot screened polypropylene (PP) cryovials or tubes are preferred for aliquots. Colored plastics and containers containing O-rings should be avoided because of the increased risk of trace element contamination from pigments. In other cases, containers used should be pre-cleaned, and the Pb transfer of the material constituting the container for the sample should be prescreened before sample collection (Salles et al. 2021). After urine collection, urine samples should be transported in dry ice and all urine specimens stored in a freezer (preferably at $\leq -20^{\circ}C$). If study participants perform collection of the urine sample at their homes, they must receive instructions for proper collection and storage (CDC 2018; Salles et al. 2021).

Saliva

Saliva is an useful alternative sample matrix for human biomonitoring. The use of this biomarker for lead offers the advantage of easy collection, noninvasive methods and dispenses with the need for medical staff. Environmental or occupational lead exposure can be assessed using this biomarker (Shawahna et al. 2021). However, several factors must be taken into account, including variations in ion content, changes in salivary flow, differences in saliva stimulation during collection, and the nutritional and hormonal status of the individual (Barbosa Jr et al. 2005; Michalke et al. 2015). Saliva reflects recent lead exposure, although results associating levels of Pb in saliva with lead concentrations in blood and plasma are conflicting in the literature. Nevertheless, use of Pb saliva as a biomarker may serve as an additional and/or alternative measurement for lead biomonitoring (Costa de Almeida et al. 2009; Gil et al. 2011; Michalke et al. 2015). Barbosa Jr et al. (2006) suggest that salivary lead may not be used as a surrogate of plasma or blood or as a biomarker to diagnose lead exposure for low to moderately lead-exposed populations. Other studies have reported low concentration of Pb in saliva and found no correlations between blood and saliva Pb levels (Costa de Almeida et al. 2010; Gil et al. 2011). The magnitude of blood Pb levels strongly influences the direction of the correlation with saliva (Gil et al. 2011). Given the lack of reference

values for lead in saliva, this biomarker should only be used to compare groups from the same study.

Whole saliva is mainly collected by five different methods: the draining method, the spitting method, the suction method, the rinsing method, and the absorbent method. Using the draining method or "passive drool," the subject allows saliva to drip off the lower lip into a tube. The participant should expectorate residual saliva into the tube only at the end of the collection period. Using the spitting method, saliva is allowed to accumulate in the mouth, and the subject spits it out into a test tube every 60 s. The suction method uses a small aspirator to withdraw saliva from the mouth. The rinsing method is a liquid-based collection system consisting of a mouth-rinsing solution. Finally, the absorbent method uses a swab, cotton roll, or gauze sponge, and after 2–5 min in the mouth, this is removed from the oral cavity and saliva transferred into a collection vial by centrifugation (Michalke et al. 2015). However, this last method described is not indicated for the evaluation of lead in saliva, due to the risk of lead contamination in the swab, cotton, or gauze. Also, before collection, the study participants should be asked to wash their mouths using water and fill a metal-free collection tube with approximately 5 mL of saliva. After collection of unstimulated salivary sample, these should subsequently be stored at −20 or −80°C (Costa de Almeida et al. 2009; Gil et al. 2011; Shawahna et al. 2021).

Hair

Human hair analysis can be used to investigate exposure to lead from various sources (Strumylaite et al. 2004; Nomura and Oliveira 2010; Michalak et al. 2014). The use of hair lead as a biomarker of environmental exposure to toxic elements has become common practice. Hair is inert and easier to sample and can be stored without technical problems (Nomura and Oliveira 2010; Michalak et al. 2014). This biomarker is also useful for assessing occupational exposure, particularly for medium to high levels of Pb pollution (Nouioui et al. 2019). The metal body burden of lead in hair reflects a record of relatively long periods because human hair can incorporate metals into its structure, providing a chemical calendar (Michalak et al. 2014; Nouioui et al. 2019; Li et al. 2021).

The main disadvantages of this matrix include the difficulty distinguishing endogenous (actual internal dose absorbed into the blood) from exogenous (external contamination) lead, and there is no consensus on the best method of removing exogenous Pb. Other disadvantages are variations between subpopulations of different race, age, and sex and in hair color and hair care, length of hair collected, and amount and position on the scalp, as well as variations in sample preparation and laboratory methodologies (Wolfsperger et al. 1994; Harkins and Susten 2003; Esteban and Castaño 2009; Peña-Fernández et al. 2017). Few studies provide reference values for correct interpretation and correlation between lead levels in the hair, blood, and other target tissues, a problem adding to the controversy over use of this matrix (Esteban and Castaño 2009; Peña-Fernández et al. 2017). Therefore, Pb in hair may not indicate internal exposure, and data may be insufficient to predict

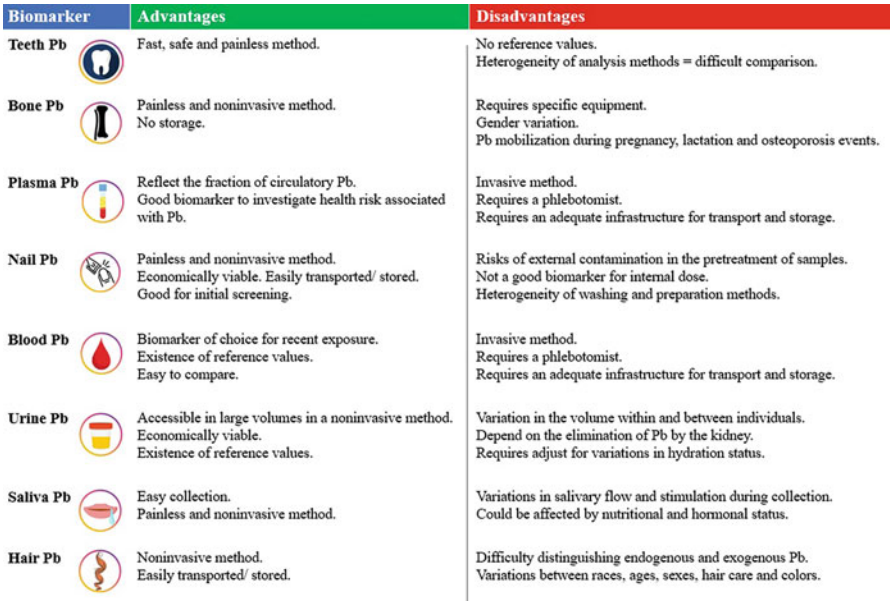

Fig. 3 Advantages and disadvantages associated with the use of each biomarker of lead exposure. This figure shows all the advantages and disadvantages of using different biomarkers of lead exposure

health effects (Harkins and Susten 2003). Especially among young girls, collecting blood, despite being more invasive, is often more acceptable than having their hair cut because children can be against cutting their hair (Personal Communication: Gouveia et al. 2014).

To collect this matrix, it is advisable to cut the scalp hair samples with stainless-steel scissors from the head as near as possible to the scalp. Different studies report collection in different regions of the head, including occipital, temporal, frontal, and cranial regions, although the occipital region is the most common site (Strumylaite et al. 2004; Michalak et al. 2014; Peña-Fernández et al. 2017; Nouioui et al. 2019; Li et al. 2021). After collection, hair samples need to be sealed in new polyethylene bags for transport and storage (Li et al. 2021). A summary of all biomarkers of lead exposure presented in this chapter considering their advantages and disadvantages is presented in the figure below (Fig. 3).

Elemental Analysis

Lead has been included in numerous human biomonitoring studies (Leroux et al. 2018; Olympio et al. 2009, 2018a, b; Oliveira et al. 2021; Salles et al. 2021). The determination of trace elements requires qualified analysts and adequate infrastructure to ensure reliable results. All steps, from sampling, storage, conservation, and

preparation, must be carefully carried out in order to avoid errors, since samples can be easily contaminated by other elements abundant in the Earth's crust (Si, Al, Fe, Ca, Mg, Na, K, Mn, and Ti) and those that are always present in the work environment, mainly in the form of dust (Zn, Pb, Cd, Hg, Cu, As, Ni) (Tölg and Tschöpel 1994). Spectrometric methods, such as inductively coupled plasma mass spectrometry (ICP-MS) and graphite furnace atomic absorption spectrometry (GF AAS), offer the sensitivity needed for trace-level quantification of Pb in biological samples. On the other hand, X-ray fluorescence (XRF) provides direct elemental determination of solids without complex sample preparation.

Studies that have used different matrices to determine lead in the human body and methods of sample preparation and analysis are presented in Table 1.

Sample Preparation

Among all of the analytical operations, sample preparation is the most critical step and the one where most mistakes occur and which is most time-consuming.

Sample preparation involves physical and chemical operations to convert the samples into a suitable form for introduction into the measuring instrument employed and to minimize interference in the quantification of the analyte. Considered a critical step in the analytical sequence because of the costs involved, time required, and high incidence of errors, sample preparation step must be strategically planned to avoid analyte losses, contamination, and costly procedures.

Throughout the analytical work, the materials used for sample preparation must be cleaned in order to prevent contamination. Plastic bottles and glassware materials must be cleaned by soaking in 10% v v^{-1} nitric acid for 24 h, rinsed with ultrapure water, and dried at room temperature. All reagents used for sample preparation and analysis must be of analytical reagent grade, the acids double sub-distilled and high purity and deionized water (resistivity 18.2 MΩ cm^{-1}). Finally, to avoid contamination from the air, the minimum that must be available is a laminar flow cabinet, but the most effective approach is to prepare the sample and solutions in a clean room.

The sample preparation of urine, blood, and plasma can be done by simple dilution with deionized water; solution of ammonium hydroxide, ethylenediaminetetraacetic acid (EDTA), nitric acid, Triton X-100, and butanol (Tanvir et al. 2020; Goullé et al. 2005); or complete decomposition with nitric acid and hydrogen peroxide under heating, following an addition dilution step with deionized water (Vorkamp et al. 2021). In the case of simple dilution, nitric acid is required to keep elements stable in the solution and Triton X-100 to solubilize the lipids of biological samples through micelle formation, lysing cells, and reducing the clogging of the introduction system of the equipment used (Freire et al. 2018). The dilution factors are important in correcting possible instrumental interference (Olympio et al. 2018a). Samples must be diluted by several factors prior to instrumental analysis. When the dilution is lower than 1: 50, instrumental calibration is usually performed using a matrix-matching approach due to high matrix interference. Aqueous calibration can be easily used for dilutions greater than or equal to 1: 50 (Olympio et al. 2018a).

Table 1 Details on different methods of preparation and analysis for different biological matrices used to determine biomarkers of lead

Matrix	Sample preparation	Instrumental analysis	LOD	References
Urine	**Dilution:** 20-fold with (0.4 v v^{-1}) HNO_3 65% and (0.005% v v^{-1}) Triton X-100	ICP-MS	n/a ; 0.13 μg L^{-1}	Freire et al. (2018) and Salles et al. (2021)
Bone	sample analyzed without previous treatment	XRF	7.6–38.63 mg $kg^{-}1$	Zhang et al. (2021)
Blood	**Dilution:** tenfold with 5 gL^{-1} of 25% ammonia, 0.5 g L^{-1} Triton X-100, and 0.5 g l−1 ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate in Millipore water	ICP-MS	0.07 μg L^{-1}	Sommar et al. (2014)
Blood	**Dilution:** 0.5% (v v^{-1}) double-distilled HNO_3 and 0.005% (v v^{-1}) Triton X-100	ICP-MS	0.05 μg L^{-1}	Costa de Almeida et al. (2010)
Blood	**Dilution:** 50-fold with a solution (0.01% (v v^{-1}) Triton X-100, 0.5% (v v^{-1}) nitric acid)	ICP-MS	10000 μg L^{-1}	Olympio et al. (2018a)
Blood	**Dilution:** sixfold with (0.2% Triton X-100 and 5% nitric acid)	GF AAS	n/a	Leroux et al. (2018)
Fingernail	**Wash:** 1% Triton X-100 and acetone **Digestion:** 10–20 mg sample, 1 ml of 25% (w v^{-1}) TMAH solution (room temperature overnight) **Dilution:** tenfold with (1% v v^{-1}) HNO_3	GF AAS ICP-MS	0.13 μg g^{-1} (ETAAS) 0.015 μg lg^{-1} (ICP-MS)	Batista et al. (2008)
Fingernail	**Wash:** acetone, HCl 0.1 mol L^{-1}, and high purity deionized water. **Digestion:** 200 μL of (65% v v^{-1}) HNO_3 in a thermostatic bath at 90 °C for 30 min **Dilution:** tenfold with deionized water	ICP-MS	1.9 μg g^{-1} 144.3 μg g^{-1}	Oliveira et al. (2021)
Fingernail	**Wash:** (0.5 % w v^{-1}) Triton X-100 and HNO_3 **Digestion:** 300 μL of 65 % (w v^{-1}) HNO_3 and 100 μL of (30 % w v^{-1}) H_2O_2 in a shaking water bath at 90 °C for 30 min or analyzing these by direct solid sampling	GF AAS	70 μg L^{-1}	Olympio et al. (2020)
Hair	**Wash:** cleaned with mild detergent and deionized water **Digestion:** 0.1 g with 4 mL (70%) HNO_3 and 2.5 mL H_2SO_4	ICP-MS	n/a	Li et al. (2021)

(continued)

Table 1 (continued)

Matrix	Sample preparation	Instrumental analysis	LOD	References
	(98%) and then heated in a sand bath			
Hair	**Wash:** cleaned with 0.1 mol L^{-1} HCl and dried by wrapping with filter paper for approximately 24 h in an oven (45 °C). A single hair strand is then cut into 36 segments of 5 mm	GF AAS	40 μg g^{-1}	Nomura and Oliveira (2010)
Plasma	**Digestion:** 100 μL of sample, 500 μL of HNO_3 **Dilution:** tenfold dilution with a 2% HNO_3 solution	ICP-MS	0.06 μg L^{-1}	Tanvir et al. (2020)
Tooth	sample analyzed without previous treatment	GF AAS	n/a	Olympio et al. (2010a, b, c) and (2018b)
Saliva	**Dilution:** 25 mL of the acid digested salivary sample, 0.75 mL of Milli-Q water, and 1.5 mL of acid diluent (1% v/v concentrated nitric acid)	ICP-MS	n/a	Shawahna et al. (2021)
Saliva	**Dilution:** tenfold with a solution containing ammonia (0.04 mol L^{-1}), (Na_2EDTA; 200 mg L^{-1}), and Triton X-100 (100 mg L^{-1})	ICP-MS	0.02 μg L^{-1}	Barbosa Jr et al. (2006)

This *table presents a list of studies using different matrices to determine lead levels in the human body and describes methods of preparation and analysis*
n/a: not available

In the case of fingernails and hair, a cleaning step is required to remove surface contaminants that can lead to overestimation of results in the analyte determination. The cleaning step must be carried out with care so as not to remove the analytes present in the samples through leaching. In the case of fingernail, the literature survey revealed many methods of washing for these samples using different reagents, such as acetone and hydrochloric acid, solutions containing Triton X-100 and nitric acid (Olympio et al. 2020), and Triton X-100 and acetone (Batista et al. 2008). Digestion procedures can be performed using a microwave oven system (Oliveira et al. 2021) or shaking water bath with nitric acid and hydrogen peroxide (Olympio et al. 2020), as well as by using tetramethyl ammonium hydroxide solution, incubated at room temperature overnight (Batista et al. 2008), followed by dilution of the sample prior to analysis.

The cleaning of hairs can be performed using different procedures: the IAEA protocol (acetone + water + acetone) (Bermejo Barrera et al. 2000), a combination of the IAEA protocol with hydrochloric acid washing (acetone + water + acetone +

hydrochloric acid) (Nomura and Oliveira 2010), and mild detergent and deionized water (Li et al. 2021) to distinguish endogenous and exogenous content. Digestion in a microwave oven system is the most common method to oxidize hair samples. Generally, nitric acid, or a mixture of nitric acid and hydrogen peroxide in various proportions, has been employed (Astolfi et al. 2020). For direct analysis of solids, dispensing with the step of sample digestion and minimizing sample manipulation and consequent contamination or analyte loss, only the cleaning step is necessary.

Measures of lead in dental enamel can be performed by surface enamel acid etch microbiopsies with 10 μL 1.6 mol L^{-1} hydrochloric acid in 70% (v v^{-1}) glycerol. The biopsy solution should be transferred to a centrifuge tube containing 200 μL of high purity water and then analyzed without previous treatment (Olympio et al. 2010c).

The Pb fraction in saliva is about 100 times lower than the dose detectable in other biological markers. These very low analyte concentrations require an analytical technique with extremely highly sensitivity, such as ICP-MS (Caporossi et al. 2010). In this case, sample dilution can be done by dilution with concentrated nitric acid (Wilhelm et al. 2002) or with ethylenediaminetetraacetic disodium dihydrate (200 mg L^{-1}) and Triton X-100 (100 mg L^{-1}) (Barbosa Jr et al. 2006).

Unlike other samples that need a digestion or dilution step, lead measurement in the bones can be carried out directly by portable XRF. This technique allows in vivo noninvasive Pb quantification within minutes (Specht et al. 2019; Zhang et al. 2021). For this approach, the area of the body to be analyzed (e.g., legs) must be cleansed before analysis. This cleansing can be done with alcohol and ethylene diamine tetraacetic acid (EDTA) cotton swabs to remove any contamination (Specht et al. 2018; Zhang et al. 2021).

Instrumental Analysis

The analytical methods to quantify biomarkers in the blood, urine, hair, fingernails, tooth, and saliva should be based on a set of selection criteria such as sensitivity, accuracy, contamination issues, and robustness. Low levels of lead, method sensitivity, and sample amount available for analysis should be considered key parameters in the selection criteria for an analytical method (Vorkamp et al. 2021).

Digested and/or diluted samples can be analyzed by GF AAS using small amounts of sample (10–30 μL) (Leroux et al. 2018), while solid samples (hair and fingernail) can be analyzed by direct sampling without previous treatment (Nomura and Oliveira 2010). GF AAS has a detection limit in the μg L^{-1} range, allowing the quantitation of most inorganic parameters in biological matrices (Michalke et al. 2015). Unfortunately, this method has several disadvantages such as a relatively small linear working range, lower analytic frequency, and, most importantly, being a mono-elementary analytic technique, although some studies involve simultaneous determinations in other applications (Luz and Oliveira 2011, 2019; Luz et al. 2013).

In the last few decades, the use of ICP-MS has been growing because of its simultaneous multielement measurement capability, coupled with much lower

detection limits at elemental concentrations in the ng/L range. Moreover, the technique offers a wider linear dynamic range, allowing the determination of major and trace elements in the same sample aliquot. A particular challenge is avoiding interference, given that ICP-MS is subject to isobaric and polyatomic interferences. Recent advances in ICP-MS technology have allowed elemental determination in different applications at trace and ultra-trace levels in complex matrices (da Silva et al. 2021). Technologies, such as kinetic energy discrimination (KED), collision/reaction cells (CRC), and triple quadrupole, can help circumvent problems with matrix and spectral interferences. Although ICP-MS can achieve an ultralow detection limit, it also has disadvantages, such as high operating and maintenance costs, need for highly specialized operator, and laboratory infrastructure.

XRF has the advantage of being a nondestructive technique, enabling direct analysis of solids. Device software is generally equipped with programs that allow elemental determination without using standards, enabling analysis of materials for which standards are not commercially available. On the other hand, XRF is subject to substantial matrix interference.

Applications to Prognosis, Other Diseases, or Conditions

Therefore, in this chapter we review analytical platforms for investigating lead exposure. From a public health standpoint, there is a major concern of a possible "silent pandemic" (Grandjean and Landrigan 2006) of neurodevelopmental disorders resulting from children's continued exposure to low lead levels (Bellinger 2008).

The form in which neurodevelopmental toxicity is expressed depends on factors such as age at exposure, coexposure to other neurotoxicants, nutritional status, genotype, and the characteristics of the home environment (Hubbs-Tait et al. 2005; Weiss and Bellinger 2006; Olympio et al. 2018a; Silva et al. 2018). The health effects of lead poisoning vary for adults and children and can be related to different concentration (μg dL^{-1}) of lead in the blood (Fig. 4).

In 2012, the CDC introduced a blood lead "reference value" (BLRV) to identify children with higher levels of lead in the blood compared to most children. This level is based on the 97.5th percentile of blood lead values in US children (1–5 years from 2015 to 2016 and 2017 to 2018 NHANES cycles). Every 4 years, the CDC reanalyzes blood lead data from the most recent two NHANES cycles to determine whether the BLRV should be updated. Children with blood lead levels at or above the BLRV represent those in the top 2.5% with the highest blood lead levels. The Federal Advisory Committee, called the Lead Exposure and Prevention Advisory Committee (LEPAC), unanimously voted on May 14, 2021, in favor of recommending that the CDC update the reference value to 3.5 μg dL^{-1} based on the NHANES data from the 2015–2016 and 2017–2018 cycles.

The CDC's BLRV is a screening tool, but the reference value is not health-based nor a regulatory standard. Once the exposure is confirmed, the person should be protected from the exposure source, the work or a given work process. Beyond the

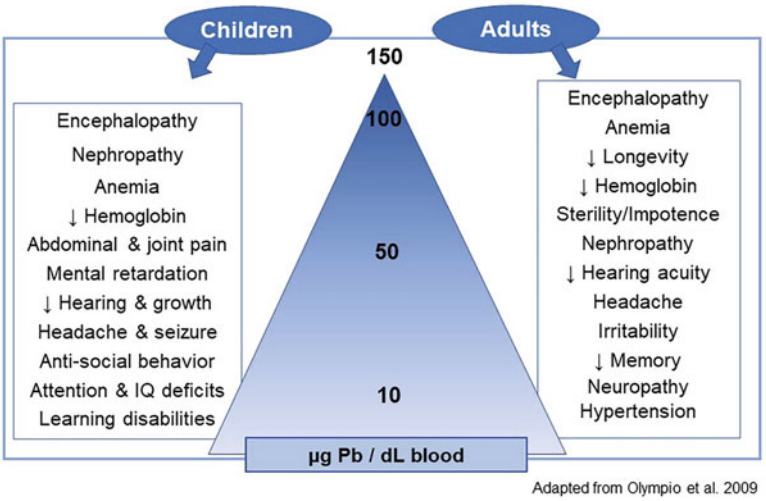

Fig. 4 Effects of lead poisoning in human health.
This figure shows different degrees of lead poisoning and their effects on human health (Adapted from Olympio et al. 2009)

environmental exposure, countries present their occupational legislations for the maximum biological index permitted for lead in workers (Brasil 1994; OSHA 2006).

Moreover, although effect biomarkers are not the focus of this chapter, in the event of a lead occupational exposure, in many cases effect biomarkers can be determined, such as blood and plasma aminolevulinic acid (ALA) levels or urinary ALA. Studies have also found many lead interference sites in the heme biosynthetic pathway. Thus, lead poisoning can be considered a chemical or acquired porphyria (Kauppinen 2005; Sassa 2006). Two thiol enzymes of the heme pathway are sensitive to lead: ferrochelatase and 5-aminolevulinic acid dehydratase (ALAD). In cases of lead poisoning, this sensitivity may cause ALA accumulation in tissues and urinary excretion (Warren et al. 1998). ALA competes with γ-aminobutyric acid (GABA), a neurotransmitter (Brennan and Cantrill 1979). Therefore, high levels of ALA in the blood and brain areas of patients carrying chemical and genetic porphyria could be associated with neurological disorders (Bechara 1996; Bechara et al. 2006).

However, focusing on lead exposure biomarkers is the action of choice because the only effective action for dealing with lead exposure is primary prevention to avoid the ubiquitous health effects of lead.

Dictionary of Terms

- ***Dental enamel.*** *Enamel is the thin outer covering of the tooth. This tough material is the hardest tissue in the human body. Enamel covers the crown, above the dentine. It is the part of the tooth that is visible outside of the gums.*

- ***Dentine.*** *The main supporting structure of the tooth, covered by dental enamel. It is 70% mineral and acellular, as hydroxyapatite crystals, and 30% organic as water, collagen, and mucopolysaccharide.*
- ***Senescence.*** *Biological aging. Natural process of gradual deterioration of functional characteristics in living organisms.*
- ***Half-life.*** *The time it takes for the concentration of a substance to fall to half of its initial value.*
- ***Creatinine.*** *Chemical compound made by muscles as part of regular, everyday activity. Filtered by the kidneys from the blood and excreted from the body in urine.*
- ***Body burden.*** *The total amount of a chemical substance in the body at any given time.*
- ***Biopsy.*** *The removal of a small tissue sample from any part of the body to examine it.*
- ***Biopsy chemical methods.*** *Calculations assume 17.4% of the dental enamel weight corresponds to phosphorus, and biopsy depth is then estimated by dividing the enamel mass (μg) per 2.95 g/cm^3 representing the density of dental enamel.*
- ***Biopsy physical methods.*** *Confocal Raman measurements acquired using a microscope with a frequency-doubled laser. The Raman spectrum of dental enamel is basically composed of hydroxyapatite peaks.*
- ***Micelle.*** *An aggregate of molecules with polar and nonpolar characteristics dispersed in a liquid.*
- ***Accuracy.*** *The proximity of measurement results or calculation to the correct value or a standard.*
- ***Robustness.*** *The ability to tolerate perturbations that might affect the statistical model, tests, and procedures. The resistance to errors.*
- ***Aliquot.*** *A portion of a solution. Material divided into small divisions.*
- ***Isobaric interference.*** *Different elements whose atoms share a common mass.*
- ***Polyatomic interference.*** *Combination of two or more atoms from different elements.*

Key Facts of Chemical Exposure

There are three common routes of exposure to a toxic substance: absorption by skin penetration, inhalation by the lungs, and ingestion by the oral route.

Occupational exposure can occur through contact with potentially toxic elements in the course of work activities.

Environmental exposure includes exposure to all chemicals present in the environment, including air, food, water, and soil.

Chronic exposure is characterized by repetitive and continuous contact with a substance over a long period (years).

Subchronic exposure is continuous exposure to a substance with an intermediate duration (months).

Acute exposure is related to a single exposure, single-dose administration, or a short contact with a chemical (hours).

Key Facts of Toxic Elements

Toxic elements are all elements that promote adverse effects at a given exposure level; some of these can also be denoted potentially toxic elements.

They are common to the environment but can be responsible for adverse health effects in organisms.

Toxic elements can be essential for the functioning of organisms or potentially harmful at a large enough concentration, while others may be potentially harmful, even at small concentrations.

The field of science that helps us understand the harmful effects exposure to toxic elements can cause is called toxicology.

Determinations of these elements in biological matrices help to establish the dose of the chemical to which a person is exposed and to investigate adverse effects.

Key Facts of Lead Poisoning

Lead is a neurotoxin and lead poisoning is recognized as a public health issue.

Pregnant women and children are the groups most vulnerable to lead poisoning.

The health effects of lead poisoning include cognitive and antisocial behavior in children and adolescents.

No level of lead exposure is considered safe to human health.

The use of lead exposure biomarkers is an approach enabling effective action for primary prevention to avoid lead poisoning.

Summary Points

- *Blood remains the biomarker of choice for recent lead exposure; despite being an invasive method, preparation for analysis can consist of a simple dilution process.*
- *Plasma lead reflects very recent exposure, as the half-life of plasma lead is shorter than 1 h. Plasma lead levels are highly elevated in the event of sudden absorption or acute exposure and rapidly diminish over time.*
- *The use of saliva as a biomarker for lead entails a noninvasive method with simple collection; however, changes in salivary flow, differences in stimulation during collection, and the nutritional and hormonal status of the individuals must be considered.*
- *Urinary lead levels reflect recent Pb absorption and depend on elimination by the kidney, which may fluctuate, and on variation between individuals, which should be adjusted by creatinine correction.*
- *Lead in nails reflects subchronic exposure and is a good tool for initial screening after removing surface contaminants; collection is noninvasive and economically viable, when other more appropriate biomarkers are precluded due to prohibitive costs or storage difficulties.*
- *Hair offers the advantage of being inert, easier to sample, and stored without technical problems; however, the use of this matrix is controversial because of the*

lack of reference values, subpopulation variations, and difficulty distinguishing internal and external exposures.

- *Teeth are recognized as biological markers of cumulative lead exposure, because of lead accumulation over embryonic and postnatal life; for example, surface dental enamel biopsies are a rapid, safe, and painless method. Given there is no reference value for lead in teeth, comparisons are valid only within the same study groups in which the same methodology was applied.*
- *Bone lead reflects cumulative exposure considering the lead concentration in skeletal burden after chronic Pb absorption; X-ray fluorescence instruments are used as a noninvasive method but are not accessible everywhere. There are variations between sexes and during pregnancy and osteoporosis.*

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Murine Embryonic Stem Cells as Platform for Toxicity Studies: Use of Human Survivin Promoter with Green Fluorescent Protein Reporter for High-Throughput Screening

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Fengli Zhang, You Li, and Shang-Tian Yang

Contents

Abstract

Embryonic and fetal development is a complicated process regulated by many signaling pathways and transcriptional factors that can be negatively impacted by drug or chemical exposure during pregnancy. It is urgent to evaluate the embryotoxic potential of drugs and emerging chemicals that are hazards to fetus. Animal models currently used in developmental toxicity evaluation are costly, inhuman, and unable to screen the enormous number of chemicals in a reasonable period. EST is the only in vitro method without sacrificing animal for studying embryotoxicity of chemicals. To address the limitations of original EST

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using morphological assessment of cardiac differentiation of murine embryonic stem cells (mESCs), novel ESTs with molecular endpoints and reporter genes have been developed for studying embryotoxicity of drugs and environmental chemicals with shortened assay duration and improved predictability. Survivin, an apoptosis inhibitor, plays a vital role in early fetal development and can thus be used as a molecular marker for screening embryotoxic chemicals. mESCs expressing enhanced green fluorescent protein (EGFP) driven by a human survivin promoter were cultured in 3D microbioreactors for screening embryotoxic chemicals. This survivin-EGFP reporter assay provides nonintrusive monitoring of cell responses to chemicals in real time with cell response dynamics that can be more reliably used in assessing toxicity. The human survivin promoter with EGFP reporter in engineered mESCs provides a fast and reliable screening of embryotoxic chemicals. The 3D EGFP reporter platform can be expanded to a multiple-endpoint system with promoters of other genes involved in various embryonic development pathways.

Keywords

Embryonic stem cell · Survivin · Developmental toxicity · Embryotoxicity · Green fluorescent protein · High-throughput screening · Luciferase · Reporter gene assay · Telomerase reverse transcriptase · 3D culture

Abbreviations

2D	Two dimensional
3D	Three dimensional
AFP	Alpha-fetoprotein
CMV	Cytomegalovirus
Cmya 1	Cardiomyopathy associated 1
CNS	Central nervous system
EB	Embryo body
ECVAM	European Center for the Validation of Alternative Methods
EGFP	Enhanced green fluorescence protein
ESC	Embryonic stem cell
EST	Embryonic stem cell test
FACS	Fluorescence activated cell sorting
FDA	Food and Drug Administration
GFP	Green fluorescence protein
Hand 1	Heart and neural crest derivatives expressed transcript 1
HTS	High-throughput screening
IAP	Inhibitor of apoptosis protein
ICH	International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use
IGF	Insulin-like growth factors
iPSC	Induced pluripotent stem cell
LIF	Leukemia inhibitory factor

mESC	Murine embryonic stem cell
MM	Micromass
OECD	Organization for Economic Cooperation and Development
PET	Polyethylene terephthalate
PSC	Pluripotent stem cell
Sox2	Sex determining region Y-box 2
TERT	Telomerase reverse transcriptase
Tubb3	Tubulin beta-3
WEC	Whole embryo culture

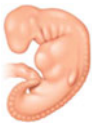
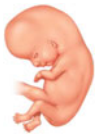
Introduction

Human body development involves a complicated process consisting of several distinct stages. At the beginning, oocyte is released into fallopian tube and then fertilization occurs when the sperm cell penetrates and passes chromosomes to the egg. Zygote undergoes through cleavage immediately to have totally 16 blastomeres. The morula enters the uterine cavity and continues division. The outer layer of blastocyst releases enzymes to enhance implantation and establish diffusion between mother and the blastocyst. Then embryonic development begins. Since around 8 weeks after fertilization, the embryo is called a "fetus," in which stage all major body organs are developed. Table 1 lists the key human fetal development stages and events before birth. After birth, human body grows through childhood and puberty until adulthood with sexual maturity.

Alterations by chemicals in the process before birth may cause developmental toxic effects, leading to birth defects. Developmental toxicity has four main manifestations: the death, structural abnormality, altered growth, and functional deficits, which might occur depending on the exposure and dose level. Developmental defects are serious health problems for human beings, among which 3% are attributed to environmental agents and 25% are caused by the combination of genetic and environmental factors such as chemical and biological agents. In 1950s thalidomide caused over 10,000 children with birth defects (Vargesson 2015), which reminds us that toxicity tests focusing primarily on adults are insufficient for understanding the drug effect on embryos or fetus. Developmental toxicity tests of chemicals including pharmaceuticals and environmental contaminants with human exposure potential are thus of significant importance.

At present, only in vivo studies with vertebrate animal models have been approved by FDA for embryotoxicity and developmental toxicity evaluation. For each chemical evaluated in animal-based studies, up to 800 animals may be sacrificed, which is not only inhuman but also costly and time-consuming. Therefore, it is imperative to develop a rapid and predictive in vitro assay platform to replace vertebrate animal models for developmental toxicity assessments. This chapter provides a brief review on limitations of animal models and current in vitro assays for developmental toxicity assessments, followed by discussions on mESC-based reporter gene assays using survivin and other molecular markers for high-throughput screening of embryotoxic chemicals.

Table 1 Stages of human fetal and body development

Stage	Developmental Events
Before birth (embryonic/fetal development)	
Germinal  (0–2 weeks)	Conception – The sperm fertilizes the egg to form zygote After a few hours, zygote begins the journey down the fallopian tube At ~24–36 h, cell division begins, and differentiation starts at 8-cell point; two distinct cell masses: Outer (to become the placenta) and inner (to form the embryo). At 1 week, cells reached uterus and developed into a **blastocyst** made up of three germ layers: 1. **Ectoderm**: To develop into skin, pigment cells, and neurons system 2. **Endoderm**: To develop into hepatocytes, lung cells, pancreatic cells 3. **Mesoderm**: To develop into cardiomyocytes, blood, bone, muscle cells Implantation – Blastocyst reaches uterus and attaches to uterine wall
Embryonic (3–8 weeks) 	Week 3 – Embryo in uterus continues to develop Week 4 – Heartbeat begins; arm buds, liver, pancreas, gall bladder, and spleen appear or start to form Week 5 – Leg buds and hand paddles appear; blood circulation begins; facial features and eyes start to form Week 6 – Fingers and toes form; lungs start to form Week 7 – Hair follicles start to form; visible elbows and toes Week 8 – Human face begins to appear; external ears start to form Cell differentiation mostly completed by the end of this period
Fetal (9 weeks to birth) 	The fetus continues to develop body systems and structures Weeks 9–15: Reflexes emerge; reproductive organs form. All parts of the body are formed by the end of the first trimester Weeks 16–25: Stronger heartbeat; body systems further developed Third trimester – Birth: Continues to develop with rapid weight and body fat increases; lungs begin to expand and contract Bones complete development Brain is continuously active
After birth	
Infancy and childhood	This stage covers the growth processes before puberty (age 10–11 for girls and age 11–12 for boys), including neonatal period, infancy, childhood, and juvenility
Puberty	Puberty is initialized by hormones from the brain to ovaries/testes. A child matures into an adult with sexual reproduction capacity in puberty
Adulthood	At adulthood, the body reaches sexual maturity

Limitations of Animal Models

Currently animal model studies are applied to evaluate developmental toxicity according to guidelines for drugs and chemicals from ICH and OECD (Festag et al. 2007). Prior to clinical trials, the US FDA requires testing in animals for pharmacologic activity and acute toxicity; therefore, laboratory animals are used for the preclinical developmental and reproductive toxicity studies to evaluate the potential clinical toxic effects of drugs (Van Norman 2019). However, this procedure usually requires a large number of animals. It has been shown that more than 12.1

million animals were used in Europe in the year 2005, of which around 0.5 million were used for safety-related assessment (Langley and Farnaud 2010). The application of animal models for safety-related evaluation is expensive, labor-consuming, and may present moral issues. There are other limitations of animal model-based developmental toxicity predictions. As published by FDA, 92% of compounds that passed preclinical animal-based tests failed in Phase I clinical trials (Langley and Farnaud 2010). One cause of the high failure rate is the species differences. Animals and human are both incompletely understood systems and the toxicity effect of a chemical observed on one species might not be reliably used to predict the toxicity on another (Van Norman 2019).

In Vitro Assays for Developmental Toxicity Assessment

To date, several in vitro methods have been studied for animal-free evaluation of embryotoxicity. Widely used in vitro toxicology tests include the WEC test, MM test, zebrafish embryo assay, and EST (Genschow et al. 2004). Table 2 lists the in vitro assays for developmental toxicity with their respective advantages and limitations.

Table 2 Alternative models and in vitro assays for developmental toxicity assessment

In vitro model/ assay	Advantages/Limitations	Accuracy	References
Rat micromass (MM) test	Detect the formation of cell foci; multiple cell functions could be observed Species-specific difference	70%	Genschow et al. (2002)
Rat whole embryo culture (WEC) test	Various endpoints are measured Species-specific difference Some effects may not be detected inside the narrow time window for gestation (day 10–12)	80%	Genschow et al. (2002)
Zebrafish embryotoxicity test	Rapid development with the organogenesis of most organs completed in 5 days post-fertilization Non-mammal with large inter-species variations	72%	Chapin et al. (2008)
Frog embryo teratogenesis assay	Use rat liver microsomes to induce metabolism Mortality, malformation, and growth inhibition as endpoints Non-mammal with large inter-species variatio Ns	88%	Bantle et al. (1989)
Mouse embryonic stem cell test (mEST)	No animal sacrificed Requires two cell lines and 3 endpoints for prediction No fetal-maternal interactions Species-specific difference	78%	Genschow et al. (2002)

MM Test

The micromass test is another in vitro method used to detect the interference of chemicals on embryo's normal cell differentiation process (Flint 1993). Embryonic limb mesenchyme or CNS cells from chicken, mouse, or rat are used to detect whether chemicals inhibit the formation of cell foci. When limb bud cells are cultured at high densities, these cells differentiate into foci of chondrocytes and neurons; therefore, multiple cell functions could be observed. However, there are some limitations of MM test on the classification of developmental toxicity. For instance, MM test gave a false-negative prediction on the strongly embryotoxic drug methotrexate, which might be caused by the folate supplementation in the culture medium. MM test is also poor in predicting if a chemical is non-embryotoxic or weakly embryotoxic, with close probabilities to classify into either class. Therefore, MM test has been less frequently used in the past decades (Spielmann et al. 2004).

WEC Test

The development of rat WEC opened the door to using mammalian post-implantation embryos for embryological research. In WEC technique, rat post-implantation embryos are cultured in vitro to directly observe, treat, and manipulate embryos to study embryotoxic effects and the mechanism of teratogenic agents. In WEC tests, various endpoints are measured, including the presence of heart beating, blood cell circulation through vitelline vessels, somite count, and crown-rump length (Ellis-Hutchings and Carney 2010). A scoring system is given for the developmental stages, and the total morphological score of all morphological endpoints is used for the analysis of developmental toxicity (Piersma 2006). The critical time window (gestation: from day 10 to 12) during neurulation and organogenesis provides an effective way to closely monitor the effects on embryonic development; however, this is also a limitation of WEC test because some effects may not be detected inside the narrow time window, causing a low prediction accuracy (de Jong et al. 2011; Dimopoulou et al. 2018).

Zebrafish Embryo Assay

Instead of using vertebrate, zebrafish assay, which monitors the adverse effect of chemicals on zebrafish embryos, has also been used in teratogenicity studies (Panzica-Kelly et al. 2012; Teixidó et al. 2013). The advantages of zebrafish embryo-based developmental toxicity assay include: small embryo models that require small volume of chemicals to test, external fertilization for easy observation of normal and abnormal development in living embryos, and rapid development as the organogenesis of most organs is completed in 5 days post fertilization, all of which make zebrafish increasingly used in toxicological studies (Ali et al. 2011; Lardelli 2008). In

zebrafish developmental toxicology assay, dechorionated gastrula-stage embryos are exposed to chemicals and then allowed to develop into the larva stage. After 5 days post-fertilization, the larvae are examined for viability and morphological integrity, and a score system is then applied for developmental toxicity assessment (Panzica-Kelly et al. 2012). However, there are many differences between zebrafish and human body systems. For instance, zebrafish is not a mammal and does not have placenta, cardiac septa, limbs, lungs, etc. (Ali et al. 2011; Lardelli 2008). Large inter-species variations may result in faulty predictions. Therefore, zebrafish embryo assays may not correctly predict toxic effects of some drugs on human.

Frog Embryo Teratogenesis Assay

Frog embryo is used for teratogenesis assay, usually with rat liver microsomes to induce metabolism to increase the predictability with mortality, malformation, and growth inhibition as endpoints (Bantle et al. 1989). The accuracy for groundwater samples containing contaminated chemicals was reported to be ~88%. This assay has the potential to detect developmentally toxic chemicals in aquatic samples and can be used to establish water quality criteria for human health and the preservation of species. However, frog is not a mammal and the large inter-species variations may limit its application in drug screening.

Embryonic Stem Cell Test (EST)

Among all in vitro methods, embryonic stem cell test (EST) is the only one that does not use pregnant animals or animal-derived tissues. The EST uses D3 murine embryonic stem cells (mESC) originally isolated from inner cell mass during early embryonic development and 3 T3 fibroblasts (Spielmann et al. 1997). ESCs are highly pluripotent with the capability to differentiate into all three germ layers and various cell lineages. They are sensitive to stresses caused by chemicals that may affect its unlimited proliferation capability and pluripotency. ESCs are thus increasingly used in developmental toxicity assessment of chemicals (Liu et al. 2017; Luz and Tokar 2018; Rezvanfar et al. 2016). EST involves three independent assays each generating one endpoint: $IC_{50\ 3T3}$ (cytotoxic effect on 3 T3 fibroblasts), $IC_{50\ D3}$ (cytotoxic effect on undifferentiated D3 cells), and ID_{50} based on morphological analysis of contracting cardiomyocytes in differentiating embryo bodies (EB) derived from D3 cells. The differentiation of ESCs into cardiomyocytes was chosen to use in EST because heart was the first organ during organogenesis, in vitro cardiac differentiation involved relatively standardized procedures, and contracting cardiac cells could be observed with a light microscope. These three endpoints are used to fit with three empirical equations respectively in a prediction model (Table 3), which is then applied to classify tested chemicals as strongly, weakly, or non-embryotoxic (Scholz et al. 1999a, b). Twenty compounds with known in vivo developmental toxicity were selected to validate the EST by ECVAM, and the results

Table 3 Linear discriminant functions and classification criteria for the EST prediction model

Linear discriminant functions	
Function I	$5.92\log(IC_{50\ 3T3}) + 3.50\log(IC_{50\ D3}) - 5.31\frac{IC_{50\ 3T3}-ID_{50}}{IC_{50\ 3T3}} - 15.7$
Function II	$3.65\log(IC_{50\ 3T3}) + 2.39\log(IC_{50\ D3}) - 2.03\frac{IC_{50\ 3T3}-ID_{50}}{IC_{50\ 3T3}} - 6.85$
Function III	$-0.125\log(IC_{50\ 3T3}) - 1.92\log(IC_{50\ D3}) + 1.50\frac{IC_{50\ 3T3}-ID_{50}}{IC_{50\ 3T3}} - 2.67$
Classification criteria	
Class 1	Non-embryotoxic if I > II and I > III
Class 2	Weakly embryotoxic if II > I and II > III
Class 3	Strongly embryotoxic if III > I and III > II

Adapted from Seiler and Spielmann (2011)

showed an overall accuracy of 78% (Genschow et al. 2000; Genschow et al. 2004; Seiler and Spielmann 2011).

Although EST can be used to evaluate developmental toxicity, its long assay duration (10 days) and quantification based on morphological assessment of contracting cardiomyocytes limit its high-throughput screening (HTS) applications (Aikawa et al. 2014). Moreover, developmental toxicity involves complicated mechanisms as chemicals can cause defects in heart, respiratory and nervous systems, chromosomal aberrations, and musculoskeletal anomalies (Petrini et al. 1997). Therefore, the assessment of cardiac differentiation could not correctly predict chemical effects on the development of other organs. EST can be improved with multiple differentiation endpoints targeting additional linages such as nervous and skeletal systems (Bremer and Hartung 2004; de Jong et al. 2014; Theunissen et al. 2013).

Reliability of measurement is another concern of EST. The judgment of the morphological endpoint for ID_{50} (contracting myocardiac cells) is challenging, which often requires the person to be highly skillful on the endpoint identification (Suzuki et al. 2011). To overcome this problem, Seiler et al. (2004) developed FACS-EST using protein markers specific for developing heart tissue to shorten the assay time to 7 days with a better quantitative endpoint. EST with molecular endpoints via proteomics (Zhang et al. 2016), transcriptomics (Theunissen et al. 2013), and gene expression profiling analyses (Van Dartel and Piersma 2011) have also been reported with significantly shortened assay time of ~5 days (Romero et al. 2011).

EST with Molecular-Biological Endpoints The establishment of real-time PCR helps to improve the developmental toxicity prediction based on molecular or biological endpoints (zur Nieden et al. 2001). Panzica-Kelly et al. (2013) developed a molecular EST with mouse D3 ESCs using the cytotoxicity IC_{50} values and transcriptional expression changes of 12 developmentally regulated genes as primary endpoints. Compared to EST that uses morphological endpoints, molecular-biological endpoints including the quantification of mRNA expression level and changes in protein expression are more objective and accurate and can be completed

in a shorter time period of 4 to 5 days. Modified ESTs using gene or protein biomarkers specific to certain differentiation lineages such as neural cells (Kobayashi et al. 2017), osteoblasts (Sittner et al. 2016), and cardiomyocytes (Suzuki et al. 2011) have also been developed. However, the isolation and reverse transcription of mRNA at different dosages of drugs and chemicals are time and labor consuming. Monoclonal antibodies for specific protein markers required in FACS may not be readily available. Moreover, the detection of molecular endpoints usually involves a sophisticated flow cytometer or qRT-PCR, which is expensive to use in high-throughput screening. In addition, the molecular endpoint assay does not provide dynamic data needed for evaluating the temporal effects or cell responses to chemicals at different time points.

mESC-Based Reporter Gene Assays

Reporter gene assays with easy-to-detect signals for high-throughput screening are widely used in drug discovery and elucidating disease-associated signaling pathways and drug action mechanism (Li et al. 2022). As illustrated in Fig. 1, a gene reporter system usually consists of a promoter sequence for the targeted gene and a reporter gene. In the assay, cultured cells with the promoter stimulated or repressed by drugs or environmental stress increased or decreased the expression levels of the reporter gene product, which are measured via the emitted fluorescence from the green fluorescent protein (GFP) or the bioluminescence from the luciferin after reaction with the luciferase released from cells. ESCs have been engineered with reporter genes for toxicology studies. Undifferentiated ESCs were transfected with a reporter gene which is controlled by a promoter associated with genes involved in tissue-specific differentiation. EST with luciferase and GFP as reporters have been developed for assessing embryotoxic potential of chemicals. In general, GFP-emitted fluorescence in cell culture is not as strong as the bioluminescent signals generated from luciferase reporters, and its detection usually uses more sophisticated flow cytometry or digital imaging microscopy, instead of conventional spectrofluorometer and fluorescent plate reader.

EST with Luciferase Reporter Gene

The most commonly used reporters are luciferases, which produce luminescent products that can be sensitively quantified. Suzuki and colleagues established ESCs with luciferase reporter gene expression controlled by cardiac differentiation markers, Hand 1 and Cmya 1, or neural differentiation markers Tubulin beta-3 (Tubb3) and Reelin (Reln) to study developmental toxicity of drugs and chemicals (Kobayashi et al. 2017; Le Coz et al. 2015; Suzuki et al. 2011). The time for embryotoxicity measurement during cardiac differentiation is reduced from

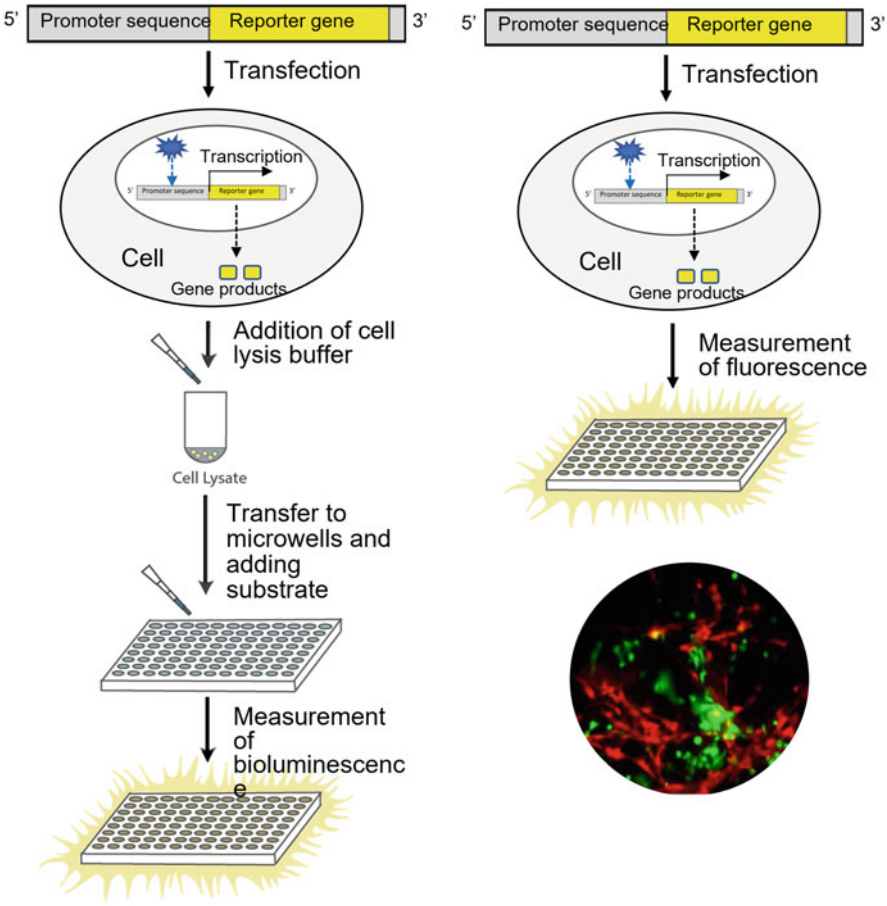


Fig. 1 Steps involved in reporter gene assays with luciferase (left) or green fluorescent protein (right) as the reporter. The fluorescent cell image shows cells expressing green and red fluorescent proteins in a coculture. Fluorescent proteins, such as EGFP, do not require additional substrates and can be used for nonintrusive, real-time detection of cell responses to drugs. Without signal amplification the fluorescent protein reporters may not be as sensitive as the bioluminescent signals in the luciferase assay and usually must be analyzed with flow cytometry or using more sophisticated digital imaging microscopic systems

10 days for traditional EST to 5 ~ 6 days using the luciferase system. However, in their studies, the addition of Steady-Glo Luciferase assay system and incubation for 2 h were required for each ID_{50} measurement, which was costly and inefficient for use in high-throughput embryotoxicity screening and could not provide real-time data because of the endpoint detections. There are also noises in measured intensity causing discrepancies in detection and actual values, such as the absorption of luciferin and the transparency of medium. The addition of substrates for bioluminescence emission is intrusive and limits the luciferase reporter assay to end-point detection, which would be costly to apply for HTS.

EST with GFP Reporter Gene

GFP driven by a cardiac-specific promoter, the α-actin promoter, was first constructed to investigate cardiac-specific differentiation during embryonic development. Results indicated that tissue-specific labeling is a powerful tool for embryological and functional investigation (Kolossov et al. 1998; Fleischmann et al. 1998). This constructed system was then applied for developmental cardiac toxicity assay. The measurement of GFP expression by flow cytometry provided appropriate toxicological endpoints for predicting chemical's effects on developing cardiac cells, making it possible for the quantitative assessment of drug and chemical effects on one specific cell lineage. However, the experiment with multiple concentrations of drugs with each concentration on one 24-well plate is labor and time consuming. GFP expression was analyzed by sophisticated FACS after embryo bodies (EBs) were digested into single cells. In addition, 50% inhibition of GFP expression was defined as IC_{50}, while the differences between cytotoxicity and embryotoxicity of drugs were not considered. Apart from cardiogenesis, GFP expression driven by AFP enhancers and promoters was also applied to study drug and chemical effects on endoderm development (Paparella et al. 2002). However, the image-based quantification analysis for AFP-GFP expression and EB sizes may cause person-to-person measurement errors, and only endpoints data are measured. In addition, image analysis is difficult for monitoring cell status in 3D cultures.

EGFP as an autofluorescent protein does not require substrates or cofactors for fluorescence detection and thus can be used as the reporter for nonintrusive detection without disrupting cell growth in the culture. However, without signal amplification the EGFP fluorescent light intensity may be low and difficult to detect by a fluorescent plate reader. On the other hand, fluorescence detection by flow cytometry or sophisticated digital imaging microscopy is cumbersome for HTS applications (Daghero et al. 2019). To overcome this detection limitation, Zhang and Yang (2011) first engineered mESCs with the EGFP gene reporter driven by a strong constitutive CMV promoter and used the reporter cells cultured in novel 3D microbioreactors to study cell proliferation/death as affected by culture conditions and drugs. Figure 2 illustrates the novel microbioreactors with PET scaffolds for 3D cultures that can be used in high-throughput drug screening. Compared to 2D cultures in multiwell plates, mESCs cultured in porous PET scaffolds grew to a higher density with more tissue-like morphology (cell aggregates or spheroids), which gave representative results and better prediction of in vivo drug effects (Langhans 2018; Li and Yang 2001; Ravi et al. 2015; Rodenhizer et al. 2018). Previous studies have also shown that the fibrous PET matrix had profound effects on cell proliferation, differentiation, and responses to drugs (Yang et al. 2005; Zang et al. 2016). Moreover, GFP-cells cultured in 3D PET matrices showed two- to three-fold higher fluorescent signals, which were amplified by the highly porous scaffold through its light focusing effect, and significantly improved signal-to-noise ratio (Xin et al. 2019), facilitating direct online detection with a common laboratory spectrofluorometer or fluorescence plate reader. On the other hand, in conventional viability assays and microscopic image analysis, variations in the size of spheroids or aggregates in 3D cultures would cause

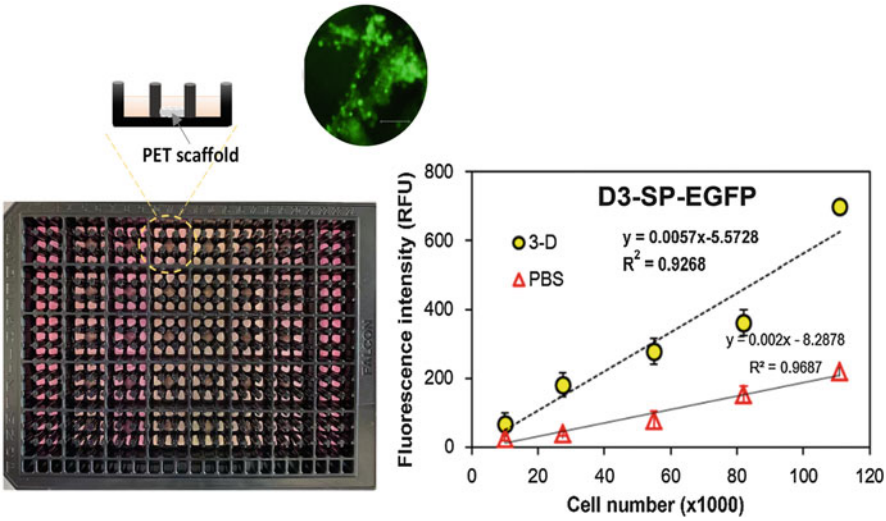


Fig. 2 Microbioreactors on a modified 384-well plate with 9 wells as one microbioreactor with a PET fibrous disk placed at the center well for 3D culture. The microscopic fluorescent image shows the EGFP-cells in the fibrous matrix. The correlation of cell number with the fluorescence intensity shows the 3D culture with three-fold higher fluorescence signal compared to the same number of cells present in PBS buffer solution. (Reproduced from Zang et al. 2019)

large measurement errors (Booij et al. 2019; Zanoni et al. 2016), which can be minimized using the fluorescent signal as the reporter. Furthermore, multicolor fluorescent cells could be used for simultaneous detection of multiple molecular markers in high-throughput drug screening as demonstrated recently by Li et al. (2022).

mESCs for HTS of Embryotoxic Chemicals

New assays with greatly improved reliability and efficiency for fast and accurate detection of embryotoxic chemicals or developmental toxicants are needed. A dynamic ESC-based HTS method is promising for rapid, reliable, and cost-effective detection of embryo toxicants. Zang et al. (2019) first developed a high-throughput embryotoxicity screening using mESCs expressing EGFP driven by a CMV promoter and a human survivin promoter, respectively. ESCs with EGFP expression driven by the CMV promoter were used as the control or reference cells to study the cytotoxicity of chemicals, whereas mESCs expressing EGFP driven by the human survivin promoter were used to assess the downregulation of survivin by chemicals. These mESCs were cultured in PET scaffolds in separate wells on a multiwell plate and EGFP fluorescence signals from these wells were monitored. The predictability of this survivin-based embryotoxicity assay was validated with chemicals with known developmental toxicity. More details are described below.

Survivin as a Biomarker for Apoptosis Inhibition and Embryotoxicity Screening

Apoptosis is one important physiologic process in almost all tissues to remove the damaged cells; however, there is no cell death found in early embryonic development due to the inhibition of the apoptotic pathway. Survivin is a member of IAPs (Ambrosini et al. 1997), which bind caspases to inhibit their mediation of apoptosis. As a bifunctional protein, survivin can inhibit apoptosis as well as regulate cell division and proliferation (Kawamura et al. 2003; Ryan et al. 2006). As shown in Fig. 3, the intrinsic apoptotic pathway through Caspase-8 or extrinsic apoptotic pathway through Caspase-9 converge to activate effector caspases and induce apoptosis. Survivin can inhibit both Caspase-9 and effector caspases, and therefore, inhibit apoptosis (Chen et al. 2016). In animal models, survivin was shown to be essential in regulating adult mouse folliculogenesis and oogenesis (Jiang et al. 2014). In another study, pregnant women were found to have a higher survivin expression level than that of nonpregnant women (Fujino et al. 2008). An earlier study confirmed that survivin was biologically important for oocyte development and maturation. Balakier et al. (2013) also reported that survivin played a critical role in normal embryo development. Furthermore, high-level survivin expression in embryo was found to correlate with better embryo quality and faster cleavage rate in animal studies. Since the disruption of survivin expression would be lethal in early-stage embryo development, survivin up or downregulation by drugs during pregnancy would likely lead to fetus anomalies.

Furthermore, survivin is highly expressed in undifferentiated ESCs, their derived tumors (Balakier et al. 2013; Blum et al. 2009; Guo et al. 2008), and cancer cells

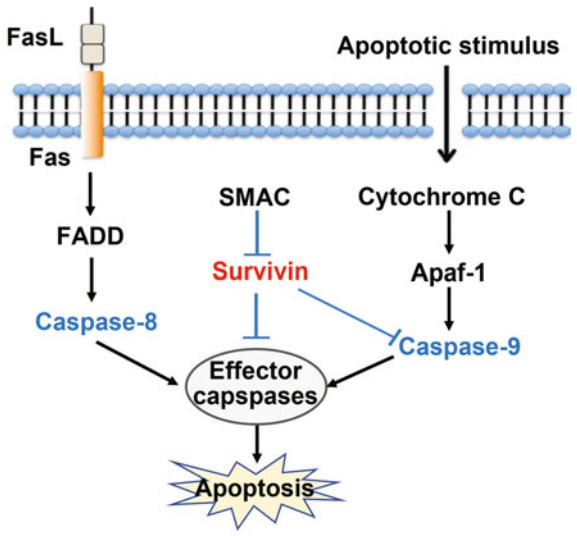


Fig. 3 The inhibition of apoptosis by survivin through repressing Caspase-9 and effector caspases, which induce apoptosis in cells. (Reproduced from Chen et al. 2016)

(Altieri 2008) but is rarely expressed in normal (mature) or fully differentiated tissues (Velculescu et al. 1999). The embryonic-specific expression of survivin makes it a good biomarker for embryo development and survivin downregulation in ESCs could be used as an indicator of embryotoxicity. Recently, Zang et al. (2019) showed that survivin expression in mESCs was drastically reduced upon differentiation (Fig. 4). Using mESCs with EGFP expression controlled by a human survivin promoter, they then showed that the specific survivin expression was significantly downregulated by known embryotoxic chemicals like retinoic acid (RA) and boric acid (BA), which inhibited the differentiation of ESCs into cardiomyocytes (Scholz et al. 1999a; b), at non-cytotoxic concentrations (0.3 µg/L and 100 mg/L, respectively); whereas non-embryotoxic saccharin at 0.18 g/L did not downregulate survivin expression (Fig. 5). The results are in accordance with our knowledge that chemicals at non-cytotoxic levels could be harmful to early embryo development and support the hypothesis that survivin can be used as a biomarker for the assessment of developmental toxicity of chemicals in a high-throughput manner. The

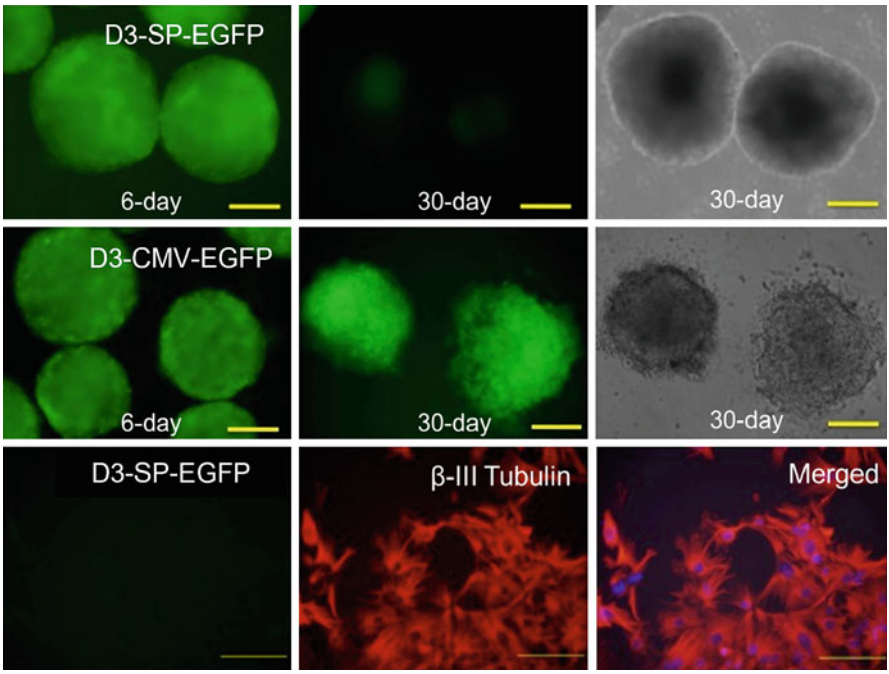


Fig. 4 Microscopic fluorescent images of EBs formed by mESCs engineered with EGFP expression. Top row shows EBs (D3-SP-EGFP cells) with EGFP expression driven by a human survivin promoter at 6 days and 30 days. Middle row shows EBs (D3-CMV-EGFP cells) with EGFP expression driven by the cytomegalovirus promoter at 6 days and 30 days. Bottom row shows the fluorescent images of fully differentiated D3-SP-EGFP cells stained with the neural differentiation marker ß-III tubulin. Scale bar: 100 µm. (Reproduced from Zang et al. 2019)

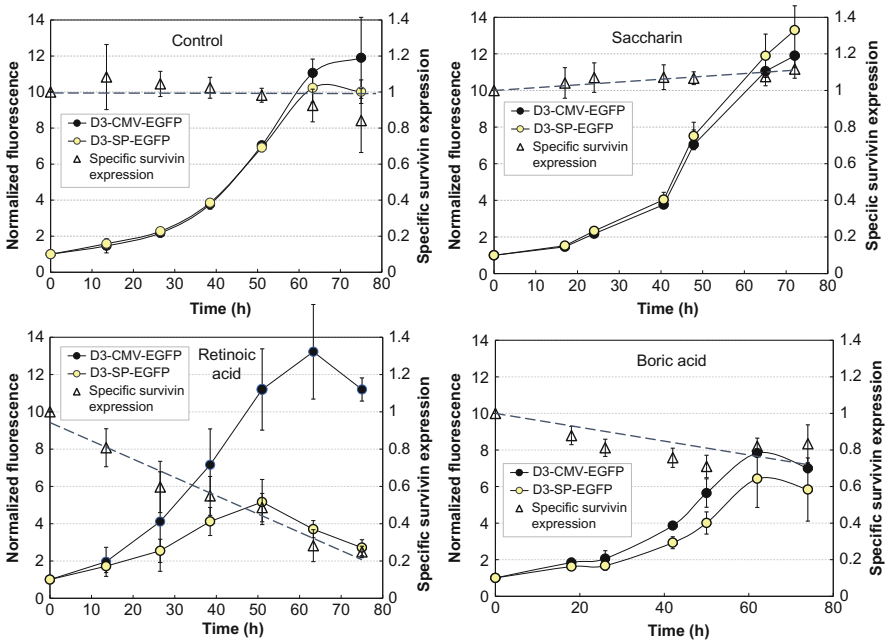


Fig. 5 Survivin expression in ESCs as affected by compounds with different embryotoxicity: non-embryotoxic saccharin, moderately embryotoxic boric acid, strongly embryotoxic retinoic acid, and no drug as control. The specific expression level of survivin remained almost unchanged during the ES cell growth (Control) and when the culture medium contained a non-embryotoxic compound (saccharin). In contrast, survivin expression was significantly downregulated in the presence of an embryotoxic compound. Black circle: D3-CMV-EGFP; yellow circle: D3-SP-EGFP; triangle: specific survivin expression (the ratio of fluorescence from D3-SU-EGFP to that from D3-CMV-EGFP). (Reproduced from Zang et al. 2019)

survivin reporter gene assay gave reliable data within 3 days, much shorter than 10 days for the EST and 6 days for the EST using specific protein markers for cardiac differentiation.

Although quantitatively monitoring the up/downregulation of survivin expression via EGFP reporter in mESCs can provide an effective method for identifying potentially embryotoxic chemicals, additional assays using molecular biomarkers specific to ESC stemness, pluripotency, and different differentiation lineages can complement the survivin assay and provide additional endpoints with new insights on the mechanisms of drug-induced developmental toxicity. Several genes involved in key signaling and embryonic developmental pathways may also be affected by chemicals, and their potential uses as biomarkers for developmental toxicity assessment. Table 4 lists some potential gene/protein markers that have been well studied and can be used in embryotoxicity screening. Some of the pluripotency and tissue-specific differentiation biomarkers are discussed further in the following section.

Table 4 Gene/protein markers for apoptosis, pluripotency, and differentiation pathways in ESC

Pathway/Lineage	Gene/protein markers	References
Apoptosis	Survivin	Zang et al. (2019)
Pluripotency	TERT Oct4 Nanog	Huang et al. (2014), Hanna et al. (2010), Young (2011)
Tissue-specific differentiation		
Endoderm	Sox17, AFP	Huang et al. (2017)
Mesoderm – Heart	Hand1, Cmya1	Suzuki et al. (2011)
	α-Actinin, MHC	Seiler et al. (2004)
Ectoderm – Neural	Tubb3, Reln	Kobayashi et al. (2017)

Other Potential Biomarkers for Embryotoxicity Screening

TERT as a Stemness Biomarker for Pluripotency Telomerase, responsible for adding TTAGGG repeats at chromosome ends and preventing telomere shortening, is closely associated with maintaining stem cell pluripotency (Huang et al. 2014). Telomerase is found to be highly active at blastocyst and embryo stages but repressed in most normal somatic tissues after undergoing cell development. Telomerase reverse transcriptase (TERT) is the catalytic subunit of telomerase, the regulation of which tightly governs the activity of telomerase (Autexier and Lue 2006). Consistently, TERT level is abundant in stem cells and germline cells but is hardly detectable in somatic cells. It was observed in a number of studies that TERT was downregulated during cell differentiation in response to differentiation inducers, like retinoic acid (Cheng et al. 2017). On the other hand, TERT's upregulation level is strongly proportional to the pluripotent state of reprogrammed iPSC (Tsai et al. 2010). TERT thus is an important biomarker to understand cell immortalization and study repression mechanism during cell differentiation, as well as initiate therapeutic interventions for limitless proliferated cancer cells (Leão et al. 2018). In stem cells, TERT was found to be directly regulated by Wnt/β-catenin signaling pathway, in which β-catenin binds to TERT promoter with the assistance of Klf4, a common transcriptional factor involved in pluripotency maintenance network (Hoffmeyer et al. 2012). Apart from the canonical telomeric function, TERT also plays a role in the regulation of mitochondrial DNA metabolism, which may further contribute to its impact on pluripotency (Teichroeb et al. 2016). Although the regulatory network of TERT is quite complex and the regulation during cell differentiation may be a systematic network and also depend on different cell context, a TERT promoter assay similar to the one with survivin promoter can be used to evaluate drug effects on stemness and thus embryotoxicity. More efforts are needed to make full use of this biomarker in stem cell and cancer research and clinical application.

Pluripotency Biomarkers Oct4, Sox2, and Nanog Stemness maintenance and self-renewal of mouse and human ESCs depend on the regulation of embryonic

development-related signaling pathways. These pathways directly control the pluripotency or collaborate with others for the maintenance of undifferentiated state. For example, the ancient and highly evolved conserved Wnt signaling pathway regulates crucial cellular behaviors during embryonic development, including cell fate, migration, polarity, neural patterning, and organogenesis (Komiya and Habas 2008). Complex extrinsic signals regulate intrinsic core transcriptional factors to eventually affect the self-renewal and pluripotency of ESCs. The maintenance of undifferentiated state can be achieved by a combination of extrinsic signals through the activation of common intrinsic transcriptional factors Oct3/4, Sox2, and Nanog (Adachi and Niwa 2013). The POU domain-containing transcription factor Oct4 is encoded by gene Pou5f1. Oct4 protein and the Pou5f1 gene have been confirmed to play vital roles in maintaining pluripotent state of ESCs by regulating various genes (Boyer et al. 2005; Chew et al. 2005; Loh et al. 2006; Zhang et al. 2006). The lack of Oct4 will cause cells with pluripotency revert to trophoblast lineages. Also, the increase of Oct4 expression above the endogenous levels could lead to the differentiation into extraembryonic endoderm lineage. Therefore, Oct4 regulates genes that participate in various cellular functions (Loh et al. 2006). Similar to Oct4, the Sox transcription factor Sox2 is also expressed in inner cell mass, epiblast, and germ cells (Avilion et al. 2003). Somatic cells reprogramming into iPSCs require Oct4 and Sox2 if endogenous Sox2 is not expressed, indicating both Oct4 and Sox2 play important roles in maintaining undifferentiated state (Hanna et al. 2010; Yamanaka and Blau 2010). The essential function of Sox2 is shown to maintain the requisite level of Oct4 expression. As found in the study using Sox2-null mouse ESCs, Sox2 together with Oct4 activated Oct-Sox enhancers to regulate pluripotency-related genes of Oct4, Sox2, and Nanog (Masui et al. 2007). The homeodomain-containing protein Nanog is a transcriptional factor that could promote a stable undifferentiated state of ESCs (Chambers et al. 2003; Loh et al. 2006; Mitsui et al. 2003). The mRNA of Nanog is present in mouse and human PSCs, but absent in fully differentiated cells. Nanog is a key downstream effector of signaling pathways such as FGF2, BMP4, TGF/Activin/Nodal, and Wnt (Mossahebi-Mohammadi et al. 2020). Intrinsically, Nanog collaborates with Oct4 and Sox2 to regulate their promoters, and form an interconnected autoregulatory loop (Young 2011). In summary, Oct4, Sox2, and Nanog as biomarkers for the pluripotent state of ESCs may also be used as additional endpoints in evaluating embryotoxicity.

Sox17 and Tubb3 as Biomarkers for Three Germ Layer Differentiations External stimulation can break the undifferentiated state of ESCs and induce the differentiation into distinct types of cells. The formation of three germ layers is a major event in the early embryonic development (Shiraki et al. 2009). The regulation of the differentiation process by compound explore can be used to assess the compound toxicity to tissue-specific differentiation. Changes in gene expression during endoderm, mesoderm, and ectoderm differentiations can be applied to assess developmental toxicity of drugs. Many gene markers during three germ layer differentiations have been studied for mouse and human ESCs. Among them, Sox17 and Tubb3 found in both mouse and human ESCs are widely studied ESC differentiation

markers. Sox17 is important for primitive mesoderm and cardiac muscle differentiation (Liu et al. 2014). Tubb3 encoding β-III tubulin would be a good neuron-specific differentiation endpoint for embryotoxicity assessment.

Conclusion

EST is the only in vitro method without sacrificing animal for studying embryotoxicity of chemicals. ESTs with molecular endpoints and reporter genes have been developed to shorten the assay duration and improve the assay predictability. Among them, engineered mESCs expressing EGFP driven by a human survivin promoter showed an enormous potential for high-throughput screening application. Signaling pathways are crucial for the fate determination of embryonic stem cells. Drug or chemical molecules may manipulate transcriptional factors by targeting the specific signaling pathways, leading to the alteration of embryonic development. The regulation mechanism that causes embryotoxicity or developmental defects is complicated and under investigation. Multiple-endpoint reporter gene assays with gene promoters of survivin, TERT, Oct4, Sox17, and Tubb3 involved in regulating apoptosis, stemness, pluripotency, endoderm, and mesoderm differentiation, as well as ectoderm neural differentiation signaling pathways may provide a comprehensive classification of developmental toxicity of chemicals. This strategy is promising for early monitoring of cellular responses to external molecule stimulation. By constructing a multiple-endpoint system with promoters of essential genes involved in important embryonic development pathways, the effects of embryotoxic chemicals on early embryonic development can be studied using the engineered mESCs with EGFP-reporters.

Summary Points

- Embryonic and fetal development is a complicated process regulated by many signaling pathways and transcriptional factors that can be negatively impacted by drug or chemical exposure during pregnancy.
- It is urgent to evaluate the embryotoxic potential of drugs and emerging chemicals that are hazards to fetus. Animal models currently used in developmental toxicity evaluation are costly, inhuman, and unable to screen the enormous number of chemicals in a reasonable period.
- EST is the only in vitro method without sacrificing animal for studying embryotoxicity of chemicals. To address the limitations of original EST using morphological assessment of cardiac differentiation of murine embryonic stem cells (mESCs), novel ESTs with molecular endpoints and reporter genes have been developed for studying embryotoxicity of drugs and environmental chemicals with shortened assay duration and improved predictability.
- Survivin, an apoptosis inhibitor, plays a vital role in early fetal development and can thus be used as a molecular marker for screening embryotoxic chemicals.

mESCs expressing enhanced green fluorescent protein (EGFP) driven by a human survivin promoter were cultured in 3D microbioreactors for screening embryotoxic chemicals. This survivin-EGFP reporter assay provides nonintrusive monitoring of cell responses to chemicals in real time with cell response dynamics that can be more reliably used in assessing toxicity.

- The human survivin promoter with EGFP reporter in engineered mESCs provides a fast and reliable screening of embryotoxic chemicals. The 3D EGFP reporter platform can be expanded to a multiple-endpoint system with promoters of other genes involved in various embryonic development pathways.

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LC-MS-Based Metabolomics in the Identification of Biomarkers Pertaining to Drug Toxicity: A New Narrative

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Abstract

The evaluation of adverse drug effects is essential for drug approval and clinical therapy. Safe and reliable biomarkers play important roles in the prediction, detection, and management of drug-induced toxicity in the preclinical and clinical studies. Identifying a highly specific, accurate, sensitive, and biologically or clinically relevant biomarker is very challenging. For instance, highly specific and accurate biomarkers for drug-induced liver injury are still not available. Nevertheless, the latest technology "omics" offer novel possibilities to develop the biomarkers pertaining to drug toxicity. Metabolomics is the systemic identification and quantification process of small biological molecules, and it serves as the downstream platform for other "omics" approaches like genomics, transcriptomics, and proteomics. Metabolomics alone, or together with other omics, has been successfully used for the identification of biomarkers in various fields, including drug toxicity. Various platforms, including nuclear magnetic resonance (NMR), gas chromatography or liquid chromatography-mass spectrometry (GC/LC-MS), and newly emerging mass spectrometry imaging (MSI), are employed in metabolomics. At present, LC-MS platform is a workhorse in metabolomics and other research fields, e.g., drug metabolism and pharmacokinetics. In some cases, drug metabolism is critical when the metabolite of a drug can be utilized as a valuable biomarker for toxicity. This review provides examples of advances in the field of metabolomics and emphasizes the application of metabolomics in biomarker discovery, drug metabolism, and mechanistic studies of drug toxicity. The challenges, opportunities, and future direction of metabolomics in biomarker discovery are highlighted as well.

Keywords

Biomarker discovery · Drug toxicity · Metabolomics · Pharmacometabolomics · LC-MS · Drug metabolism · Bioactive metabolites · Metabolite identification · Drug-induced liver injury · Metabolic pathway mapping

Abbreviations

AFB1	Aflatoxin B1
ALT	Alanine transaminase
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
APPI	Atmospheric pressure photoionization

AST	Aspartate transaminase
AUC	Area under the curve
BUN	Blood urea nitrogen
CE	Capillary electrophoresis
CI	Chemical ionization
CPB2	Carboxypeptidase B2
CPK	Creatine phosphokinase
CYP	Cytochrome P450
DILI	Drug-induced hepatotoxicity
EI	Electron ionization
ESI	Electrospray ionization
FMO	Flavin-containing monooxygenases
FT-ICR	Fourier transform ion cyclotron resonance
GC	Gas chromatography
GNMT	Glycine N-methyltransferase
GSH	Glutathione
HCC	Hepatocellular carcinoma
HLM	Human liver microsomes
HMDB	HUMAN Metabolome Database
HRMS	High-resolution mass spectrometers
IC	Ion chromatography
IPA	Ingenuity Pathway Analysis
LC	Liquid chromatography
MALDI	Matrix-assisted laser desorption/ionization
MetPA	Metabolomics Pathway Analysis
mGWAS	Metabolite genome-wide association studies
miRNAs	microRNAs
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MSI	Mass spectrometry imaging
NAc	N-acetylcysteine
NMR	Nuclear magnetic resonance
OPLS-DA	Orthogonal projections to latent structures discriminant analysis
Orbitrap	Orbital ion traps
PCA	Principal components analysis
QC	Quality control
QQQ-MS	Triple quadruple mass spectrometry
Q-TOF	Quadrupole time of flight
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
Scr	Serum creatinine
SFC	Supercritical fluid chromatography
SPE	Solid-phase extraction
TOF	Time of flight

Introduction

Efficacy and safety are the two fundamental elements for a marketed drug. Biomarkers should have objective characteristics, which can be measured accurately and reproducibly and cited to evaluate medical states like pathogenic processes or pharmacological responses to treatments. Identifying the biomarkers of drug-induced toxicity is extremely challenging as the molecular phenotypes can be highly variable in different stages and target organs. During the preclinical development stage, biomarkers also play important roles in safety monitoring (Schofield et al. 2021). Enzymes, like alanine transaminase (ALT) for liver injury, are most commonly used as biomarkers for drug toxicity, though this can sometimes be less specific (Araujo et al. 2017). Recently, cytokines (Yang et al. 2021), microRNAs (miRNAs) (Schofield et al. 2021), and small molecules (metabolites) have also been employed in monitoring drug efficacy and safety (Araujo et al. 2017). Successful biomarkers generally have high accuracy, specificity, sensitivity, biological significance, or clinical plausibility and are readily and safely obtainable (Proudfoot et al. 2011). The latest technologies, through an omics approach, have provided novel tools to develop biomarkers pertaining to drug toxicity.

The "omics" is defined as the study of the abundance and (or) structural characterization of a broad range of molecules in organisms. Omics help tie together the structures, functions, and dynamics of living creatures across many areas of study. According to the target objects, the "omics" can be generally classified into genomics (DNA), epigenomics (the epigenetic modifications of genome), transcriptomes (mRNA), proteomics (proteins), and metabolomics (metabolites) (Fig. 1) (Gorrochategui et al. 2016; Balcerczyk et al. 2020). Metabolomics, one of the omics, is the systemic identification and quantification of small biological molecules and has been successfully applied for biomarker identification in various fields, including drug toxicity. The chemical nature of the metabolome (i.e., metabolites) remains consistent in all species, while the genome, mRNA, and protein sequences significantly vary from species to species (Balcerczyk et al. 2020). Notably, the protocols for metabolite detection in murine models are readily transplanted to clinical samples (Jia et al. 2021). Thus, metabolomics is an efficient and ever-growing tool in identifying toxicity biomarkers that are specific indicators of damage to a particular organ in preclinical and clinical studies (Beger et al. 2010). This review focuses on the advances of metabolomics in biomarker discovery, drug metabolism, and mechanistic studies of drug toxicity.

Metabolomics

Metabolomics is downstream of other omics, such as genomics, transcriptomics, and proteomics, and is considered the closest node to the molecular phenotype. Metabolomics links the genotype and phenotype of an organism by associating a measurable chemical response with a biological event (Marshall and Powers 2017; Luque De Castro and Priego-Capote 2018). Metabolomics is the large-scale study

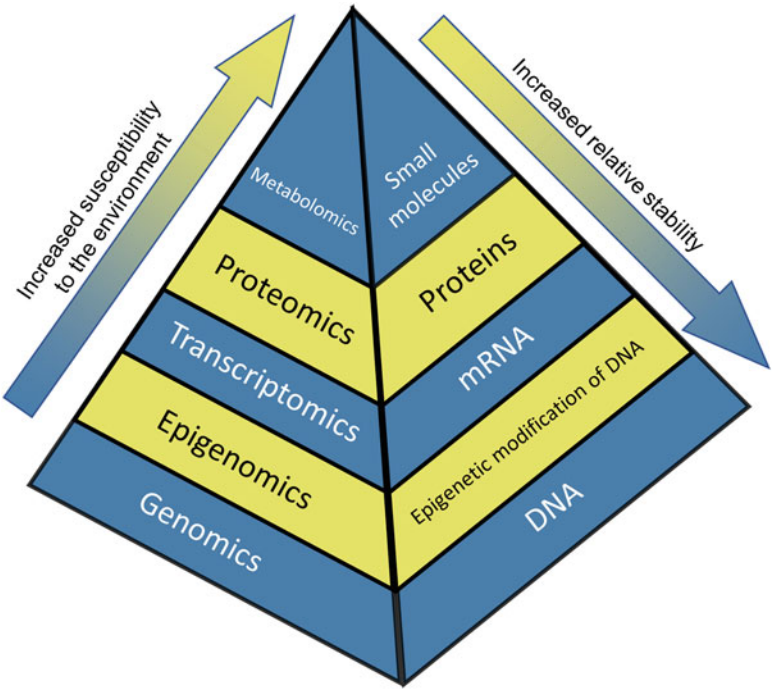

Fig. 1 Overview of "omics" platforms and their corresponding targets. Genomics, the study of the genome (the complete set of genes); epigenomics, the study of the epigenome (all the epigenetic modifications on the genetic material of a cell); transcriptomics, the study of transcriptome (the complete set of RNA transcripts); proteomics, the study of proteomes (the large-scale study of proteins); metabolomics, the study of metabolome (the large-scale study of small molecules)

of metabolites with molecular weights less than 1.5 kDa that comprise the metabolome (Nicholson and Lindon 2008; Johnson et al. 2016; Dettmer et al. 2007). Therefore, metabolomics is also considered as the implementation of various analytical platforms and instrumental techniques for the identification of metabolites and metabolic pathways in organisms (Johnson et al. 2016).

With the rapid advancement of instrumentation (e.g., nuclear magnetic resonance (NMR), mass spectrometry (MS)), development of databases (e.g., Human Metabolome Database (HMDB), METLIN, MZedDB, and the KEGG pathway), and development of analytical software (Alseekh et al. 2021; Want et al. 2013), metabolomics research bloomed in the last two decades (Fig. 2). The primary aim of metabolomics is to pinpoint the significant metabolites that contribute to system biology issues like disease pathogenesis and development, drug efficacy and toxicity, and environmental and genetic variation. (Wiklund et al. 2008).

Thus, metabolomics provides novel strategies to discover biomarkers of small molecular metabolites. Additionally, pharmacometabolomics, a subset of metabolomics, is the comprehensive study of metabolites to evaluate pharmacokinetic profile (absorption, distribution, metabolism, excretion, and toxicity) of a drug and to

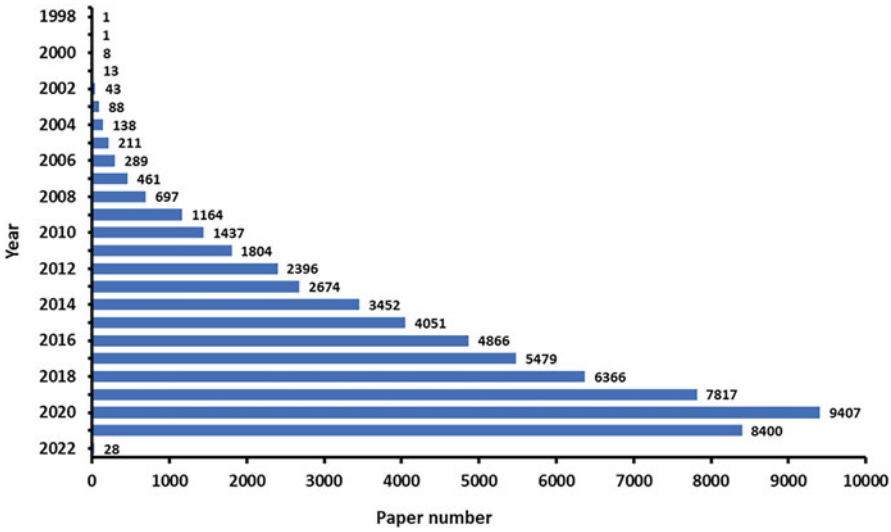

Fig. 2 Paper published from 1998 to 2022 in PubMed searched with the keyword "metabolomics." The first paper concerning metabolomics was published in 1998 and over 9000 papers in 2020

map the drug effects on pharmacodynamics (Yang 2012; Yerges-Armstrong et al. 2013). Pharmacometabolomics is well applied to biomarker identification of disease diagnosis and the mechanistic study of drug toxicity (Yang 2012; Beger et al. 2010; Beger et al. 2020).

Analytical Platforms in Metabolomics

Various analytical platforms have been used for metabolomics. NMR and MS are most frequently employed to detect and identify metabolites (Riekeberg and Powers 2017). Gas chromatography (GC), liquid chromatography (LC), capillary electrophoresis (CE), ion chromatography (IC), and supercritical fluid chromatography (SFC) are the common techniques for resolving metabolites from biological matrices (Beale et al. 2018; Van De Velde et al. 2020; Saigusa et al. 2021). Specifically, MS coupled with these separation platforms can render metabolite separation and identification more efficiently. The pros and cons of different platforms will be discussed individually in the following sections.

NMR-Based Metabolomics

^{1}H-NMR spectroscopy is widely used in metabolomics studies, as nearly all metabolites contain hydrogen atoms and could give an ^{1}H-NMR spectrum. The ^{1}H-NMR spectrum of a biological fluid sample provides the signal sum of hundreds of metabolites (Nicholson and Lindon 2008), while studies with ^{13}C-NMR,

^{31}P-NMR, and ^{15}N-NMR are also reported (Baverel et al. 2017; Wang et al. 2021a; Furse et al. 2021). NMR is a robust tool for the identification of unknown compounds, as it provides atomic-level information (Edison et al. 2021). NMR is highly automatable and exceptionally reproducible, and it is more suitable for high-throughput, large-scale metabolomics studies than some of the other techniques mentioned thus far (Emwas et al. 2019). Moreover, due to its advantageous characteristics (e.g., nondestructive, requires the least amount of sample preparation steps, has high reproducibility, and nonselective), NMR exhibits superior strength compared with other platforms in the field of in vivo and dynamic metabolomics (Guleria et al. 2018; Edison et al. 2021). However, the primary disadvantages for NMR are the relatively low sensitivity and highly overlapped signals. With limits of detection range from μM to mM in concentration and complicated spectrum interpretation, the application of NMR-based approach can be restricted to some extent (Mastrangelo et al. 2015; Marshall and Powers 2017). However, recent developments in NMR technology have improved its sensitivity significantly, in some cases to the nanomolar level (Dalisay et al. 2009; Sellies et al. 2019), and the large dynamic range of NMR data acquisition can expand the quantitative concentration range of metabolites. Nonetheless, NMR is still a few orders of magnitude less sensitive than MS in most studies (Edison et al. 2021; Marshall and Powers 2017).

MS-Based Metabolomics

MS-based metabolomics has become a "workhorse" in metabolomics studies due to the high sensitivity and large dynamic detection range from picomoles to millimoles. The approaches of MS coupled with GC or LC are extensively utilized in metabolomics studies. GC-MS is ideal for the discovery and identification of low-molecular-weight, nonpolar, and volatile metabolites (Beale et al. 2018; Siuzdak 2016). The two main ionization methods used in GC-MS are electron ionization (EI) and chemical ionization (CI), with the former one most frequently used in metabolomics to date (Beale et al. 2018; Mastrangelo et al. 2015). EI provides highly reproducible patterns of ion fragmentation in both ion m/z and relative abundance regardless of the GC instrument used (Mastrangelo et al. 2015). GC-MS-based metabolomics is the "gold standard" for volatile metabolites with low molecular weights (Pautova et al. 2021). The low-molecular-weight metabolites, like amino acids, short-chain fatty acids, steroids and their derivatives, hydroxy acids, dicarboxylic acids, and nucleosides, could be easily detected and identified using GC-EIMS and EI spectra in libraries (Wishart et al. 2008). Two-dimensional (GC × GC-MS) platforms with extremely high resolution and selectivity have been adopted in the analysis of complicated samples, like essential oil (Cordero et al. 2015), and saliva (Cialie Rosso et al. 2021) samples. Besides, the lower cost of GC equipment and readily optimized parameters and processes make GC-MS attractive in metabolomics (Pautova et al. 2021). For nonvolatile or thermolabile analytes, chemical derivatization is required before analysis, which in part limits the application of GC-MS. Chemical derivatization could complicate the final mass spectrum, because multiple derivatization products could be produced from one metabolite and present as various peaks (Beale et al. 2018).

LC-MS, which offers high sensitivity, selectivity, and reproducibility, is another frequently employed system used in metabolomics. LC-MS is capable of analyzing thousands of metabolites simultaneously in one biological sample, which is especially suitable for the detection of thermally labile nonvolatile metabolites, with a wide range from nonpolar to polar compounds (Chen et al. 2016). Currently, the atmospheric pressure ionization (API) is the most frequently used ionization method for LC-MS. API is a "soft ionization" method that generates less fragmentation, preserving the intact molecular information (Siuzdak 2016). API consists of electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization (APPI), and matrix-assisted laser desorption/ionization (MALDI), suitable for analytes that fall into different ranges of molecular weights and polarity. Of these ionization methods, ESI is widely adopted in LC-MS-based metabolomics as compatible with various analytes from relatively nonpolar to polar compounds with a high practical mass range and is easily coupled with LC instruments (Siuzdak 2016; Rajawat and Jhingan 2019). Ion suppression is an issue associated with LC-MS, particularly with ESI, which can be partially surmounted by employing nanospray ionization and improved metabolite separation. In addition, quantification of metabolites for LC-MS-based metabolomics is challenging as different metabolites can have various MS responses due to the different ionization efficiencies. Directly comparing the abundance across metabolites based on their responses (peak areas) may result in inaccuracy. Thus, relative quantification, comparing the responses of a certain analyte in the experimental groups with that in the control group, is a frequently adopted strategy in GC-MS- and LC-MS-based metabolomics studies (Alseekh et al. 2021). Currently, a number of databases of metabolite identities and structures are publicly available for LC-MS metabolomics (e.g., HMDB, METLIN), which are critical for metabolite identification (Edison et al. 2021).

To obtain a global metabolic profile of a certain sample, LC/GC-MS and NMR platforms can serve as complements for each other in metabolomics studies (Mastrangelo et al. 2015; Zeki et al. 2020). Comprehensive profiling of the metabolites is especially pivotal for associating phenotypes with certain kinds of metabolites in the mechanistic study of drug efficacy and toxicity (Zeki et al. 2020). The pros and cons of NMR-, GC-MS-, and LC-MS-based metabolomics approaches are listed in Table 1 (Saigusa et al. 2021; Lu et al. 2019; Marshall and Powers 2017; Ellis et al. 2007).

Types of Metabolomics

Metabolomics can be categorized into untargeted and targeted metabolomics based on the purposes of studies regardless of any analytical platforms. Untargeted metabolomics is an unbiased approach to detect all the possible metabolites and to relatively quantify the metabolite levels in different sample groups of interest. Untargeted metabolomics studies aim to put forward novel hypotheses or discover biomarkers based on highlighting altered metabolites and aberrant biochemical

Table 1 Comparison between NMR- and MS-based metabolomics

	NMR-based metabolomics	MS-based metabolomics	
		GC-MS	LC-MS
Structure information	Available	Limited	Limited
Sample preparation	Simple and no chemical derivatization needed	Chemical derivatization is often needed	Specific methods should be developed
Sample recovery	Available	Not available	Not available
Target metabolites	Relatively hydrophilic	Volatile	Nonvolatile
Linear range	Narrow	Wide	Wide
Sensitivity	Low (μM to mM)	High (fM to mM)	High (pM to mM)
Inter-lab reproducibility	Very high	High	Average
Across-lab reproducibility	Very high	High	Average
Quantification	Absolute	Relative	Relative
Libraries	Available	Available	Limited

fM femtomolar, *pM* picomolar, *mM* millimolar

pathways (Griffin 2020; Mastrangelo et al. 2015). It usually provides some clues and insights into exploring an unknown mechanism or biomarkers of disease or toxicity. For untargeted LC-MS-based metabolomics, high-resolution mass spectrometers (HRMS), such as time of flight (TOF), quadrupole time of flight (Q-TOF), orbital ion traps (Orbitrap), and Fourier transform ion cyclotron resonance (FT-ICR), are frequently employed as high mass accuracy of ions that is essential for predicting experimental formula required for metabolite identification (Qi et al. 2014; Gorrochategui et al. 2016).

To compare with, targeted metabolomics measure a specific group of metabolites based on the hypothesis. Generally, a more sensitive and quantitative method could be established (Beale et al. 2018) as the standards for the metabolites are available, and standard curves could be set up for the absolute quantification and linear range determination (Johnson et al. 2016; Qi et al. 2014; Alseekh et al. 2021). The absolute concentrations from targeted metabolomics studies (e.g., in the unit of μg/mL) should be independent from the quantification instrument and protocols and can be used in the comparisons among labs and sample batches (Vinayavekhin and Saghatelian 2010). Triple quadruple mass spectrometry (QQQ -MS) in multiple reaction monitoring (MRM) mode can meet the needs of targeted metabolomics, which could provide the higher sensitivity and better quantification (Vinayavekhin and Saghatelian 2010; Ellis et al. 2007). Frequently, untargeted and targeted metabolomics are concurrently used to address important biological questions. The differential aims, characteristics, and uses are summarized in Table 2 (Johnson et al. 2016; Riekeberg and Powers 2017; Gorrochategui et al. 2016; Siuzdak

Table 2 Comparison between untargeted metabolomics and targeted metabolomics

	Untargeted metabolomics	Targeted metabolomics
Prior knowledge of the metabolome	No	Yes
Hypothesis	Hypothesis generating	Hypothesis-driven
Metabolite collection	All possible metabolites	Metabolites of interest
Sample preparation strategy	Extract as many metabolites as possible	Optimized for specific metabolites
References	Databases/libraries	Reference standards needed
Quantification	Relative (peak areas)	Absolute available (e.g., ng/mL)
Selectivity and sensitivity	Average selectivity and sensitivity Average reproducibility High false-positive rate	High selectivity and sensitivity Higher reproducibility Low false-positive rate
Data interpretation	Difficult, bioinformatic tools needed	Relatively easy
Metabolite identification	Required	Known

2016). In the following section, a focus is made on the workflow and application of LC-MS-based untargeted metabolomics in drug toxicity.

LC-MS-Based Metabolomics: Workflow and Experimental Design for Drug Toxicity

LC-MS-based untargeted metabolomics plays important roles in exploring the biomarkers and mechanistic studies of drug toxicity. The general workflow presented in Fig. 3 depicts experiment design, sample collection and preparation, data acquisition, data preprocessing and analysis, metabolite identification, and data interpretation. These steps will be individually discussed in detail.

Experiment Design

A rigorous experimental design is critical for addressing a biological question. As discussed in section "Types of Metabolomics," untargeted metabolomics is hypothesis generating, while targeted metabolomics is mainly used to validate the established hypothesis. Currently, the mechanism of toxicity remains unclear for most drugs. Untargeted metabolomics could provide a global and unbiased picture for the metabolism of an organism at the molecular level (Bartel et al. 2013). The possible altered pathways involved in drug toxicity could be hypothesized building on the metabolomic findings. The required number of replicates in each group depends on the sample type, desired statistical power, effect size, and actual variance (Table 3). The comparability should be well validated in different batches of samples

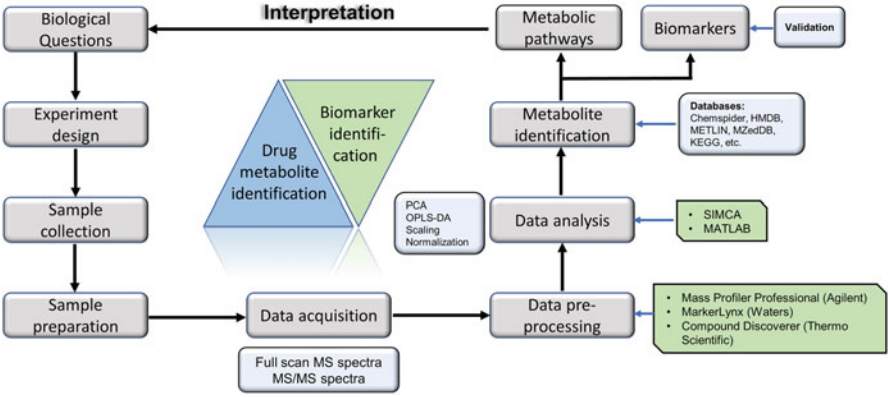

Fig. 3 A workflow for untargeted LC-MS-based metabolomics. The workflow contains experiment design, sample collection and preparation, data acquisition, data preprocessing and analysis, metabolite identification, and data interpretation. PCA, principal components analysis; OPLS-DA, orthogonal projections to latent structures discriminant analysis; HMDB, Human Metabolome Database; KEGG, Kyoto Encyclopedia of Genes and Genomes

Table 3 Recommended sample sizes for different sample resources in a metabolomics study

	Cell culture	Small animals	Human subjects
Optimal	>7	>10	>50
Rigorous	6–7	8–10	40–50
Acceptable	4–5	6–7	25–40

collected separately. As human samples are subjected to larger interindividual variation and differential genetic background, it is important to include enough numbers of samples in each group in order to draw a reliable conclusion. For cell experiments, replicated wells and independent replicates of experiments are required. Special control groups should be included for the validation of experiment outcomes (Alseekh et al. 2021).

Sample Collection and Preparation

As metabolites rapidly change in living organisms, the method of sample collection could significantly influence the results. Incorrect sample collection may result in misleading data. For solid samples (e.g., tissue, organoids, cells), snap freezing in liquid nitrogen and further storage at under −80 °C before sample preparation are recommended. For fluid samples (e.g., urine, plasma, cell medium), directly storing samples at under −80 °C is frequently employed before analysis. Storage at ultralow temperatures can generally suppress enzyme activity and avoid further degradation of metabolites. Alternatively, quenching samples with organic solvents (e.g., methanol, acetonitrile) or acids (e.g., formic acid) is also frequently used in the collection

of fluid samples since organic solvents and acids also destroy the enzymes to stop possible reactions. In principle, the collection approaches attempt to reflect the real levels of metabolites in a living organism at the collection time point. Inappropriate methods may render the loss of key metabolites. For instance, repeated freeze-thaw cycles should be avoided, as the degradation of metabolites will be accelerated under these conditions (Alseekh et al. 2021; Balcerczyk et al. 2020; Zeki et al. 2020).

For non-fluid samples, physical extraction methods are often required to release metabolites from them. Sonication and homogenization are common physical treatments for cell and tissue samples (Beale et al. 2018). For some extremely unstable metabolites, immediate extraction and analysis of samples are required. LC-MS is very susceptible to ion suppression brought by the biological matrix in the samples. Efficient removal of a biological matrix will increase LC resolution and MS sensitivity. Common metabolite extraction and matrix removal methods include protein precipitation, liquid-liquid extraction, and solid-phase extraction (SPE) (Zeki et al. 2020). However, no universal solution is available for all types of samples or to extract all metabolite classes. Moreover, excessive extraction steps will result in significant analyte loss and failure of detection for trace metabolites (Zeki et al. 2020; Michopoulos et al. 2009). To summarize, the ideal sample preparation method for untargeted metabolomics should meet the following criteria: (1) nonselective for untargeted metabolomics and selective for targeted metabolomics; (2) reproducible; (3) simple, fast, and without metabolite loss and/or degradation; and (4) high throughput (Dettmer et al. 2007; Dunn et al. 2011; Want et al. 2013; Luque De Castro and Priego-Capote 2018). The efficiency, reproducibility, and compatibility of the extraction method should be evaluated before and during the sample analysis process. Proper multiple internal standards that cover a range of chemical structure classes should be spiked into the biological samples prior to extraction for normalizing the instrument variation (Beale et al. 2018). Pooled quality controls (QC) are used to (1) monitor the quantitation quality and repeatability of a batch of samples (acceptable criteria: coefficients of variation of metabolite feature intensity within 20%), (2) to equilibrate the analytical platform before the samples are injected, and (3) to provide data to use for signal correction within and between analytical batches (Dunn et al. 2011; Want et al. 2013). QC samples are generally prepared by thoroughly mixing and extracting small aliquots of each biological sample to be studied along with samples. Overall, the sample preparation methods should be carefully chosen according to the study purposes and the nature of samples.

Data Acquisition and Analysis

The high mass resolution of MS serves as the foundation for metabolite identification and the generation of experimental formulae. To date, HRMS like TOF and Orbitrap could have a mass accuracy with errors of less than 1 ppm (Fenaille et al. 2017). The MS data from metabolomics are usually acquired in profile mode instead of centroid mode because profile mode provides the isotope distribution and continuous function, which are not available in centroid mode. Isotope fine features are

very valuable for identification of unknown metabolites. Additionally, compared with profile mode, centroid mode lost the information concerning noise characteristics, linearity of the ion signal, and mass spectrally interfering ions (Lebanov et al. 2021; Gorrochategui et al. 2016). The acquired raw MS data is in the original proprietary formats that can be processed by the corresponding software packages developed by MS manufacturers, like Mass Profiler Professional from Agilent, MarkerLynx from Waters, and Compound Discoverer from Thermo Scientific. The data can also be converted into open data formats, of which the most popular are XML-based formats that can be further processed by software like SIMCA and MATLAB (Gorrochategui et al. 2016). Briefly, raw data are first input into the matched software (provided by MS manufacturers), which undergoes preprocessing for peak detection (with certain m/z) and peak alignment of data collected from LC-MS. The peak matching and alignment enable the data in the individual samples to be comparable (including QC samples). Generally, LC-MS-based untargeted metabolomics can produce a gigabyte-sized raw data file, which makes data analysis very challenging. To date, many chemometric analytical software have been developed to analyze the high-dimensional raw data, like SIMCA-P and MATLAB. Principal components analysis (PCA) and orthogonal projections to latent structures discriminant analysis (OPLS-DA) are routinely performed for metabolomics data analysis. Unsupervised PCA is a rapid way to assess sample clustering, outliers, and class separation, in which *m/z* and retention times are usually used for analysis. Compared with PCA, OPLS-DA is a supervised analysis, which aims to figure out which variables are driving the separation between the two groups (Want et al. 2013; Worley and Powers 2016; Qi et al. 2014). The chemometric analysis will reveal the metabolites contributing to group separation, which could be the potential biomarkers (Want et al. 2013).

Following MS data analysis, identification of altered metabolites of interest is essential for biomarker discovery. Currently, determining the structure of unknown metabolites is still a bottleneck for LC-MS-based metabolomics. HRMS could produce the exact masses, which allow the prediction of putative molecular formulae and the search in the available databases, while MS/MS technology can provide more structure information from ion fragmentation. Thus, HRMS together with MS/MS fragments is indispensable for metabolite identification. To date, the publicly available databases for endogenous metabolites include HMDB, METLIN, MZedDB, ChemSpider, and KEGG. These databases are comprehensive, include synthetic and natural compounds, but are not specific for metabolomics. Moreover, MS/MS fragments are highly dependent on instruments and MS parameters like collision energy. Although some databases do offer MS/MS spectra generated from different collision energies, metabolite identification remains very challenging as the MS/MS spectra may be not comparable. In the pharmacometabolomics, the identification of unknown drug metabolites primarily depended on exact masses and MS/MS spectra. Comparing MS/MS fragments of metabolites with those of parent drugs could elucidate the putative chemical structure (i.e., the possible metabolism sites of a parent drug). In general, elucidating the exact structure of a metabolite should be performed by comparing the exact mass, retention time, and MS/MS

fragments with those of an available standard compound (Alseekh et al. 2021; Want et al. 2013).

Data Explanation and Biological Question Addressing

The biological pathways related to those altered metabolites identified could be mapped using different software, for example, Ingenuity Pathway Analysis (IPA) or Metabolomics Pathway Analysis (MetPA). IPA is the leading software for integrating and interpreting the data derived from omics experiments. MetPA is a user-friendly web server used to identify and visualize metabolic pathways based on metabolomics data and has been used to study the mechanisms of drug toxicity (Liu et al. 2020). Successfully determining the possible pathways involved with the changed metabolites is very valuable for investigating mechanisms of disease or drug toxicity. Findings from untargeted metabolomics should be validated with targeted metabolomics. For targeted metabolomics, the final data interpretation is much more straightforward as it is hypothesis-driven from the beginning. Connecting different omics will also contribute to elucidating the true mechanism. Therefore, this process is highly dependent on the purpose of the study and is very case-specific. However, in order to address the biological questions and avoid overstatement of metabolomics data, various conventional biological experiments should be designed to validate the potential metabolic checkpoints discovered in metabolomics study (Want et al. 2013).

Applications of LC-MS-Based Metabolomics for the Study of Drug Toxicity

The Roles of Metabolomics in Drug Toxicity

Drug-induced toxicity is caused by the abnormal reactions of the body to xenobiotics, which do not fall into the expected pharmacological effects. LC-MS-based metabolomics can not only monitor xenobiotics and their metabolites, especially reactive metabolites, but also analyze the changes of endogenous metabolites. Thus, unique advantages place metabolomics in a good position to identify the biomarkers and mechanisms of drug toxicity. LC-MS-based metabolomics has been successfully applied to the study of drug metabolism and shed light into the mechanisms of drug toxicity.

Screening Drug Metabolites

LC-MS-based metabolomics has been proven as a powerful tool for the profiling of drug metabolism. Both stable (MacKenzie et al. 2020) and unstable (bioactivated) (Li et al. 2011; Li et al. 2014) metabolites can be identified by this approach in the absence of synthesized standards and isotope-labeled standards. Compared with the traditional LC-MS methods, metabolomic strategy could avoid the laborious process

of predicting possible metabolites and readily identify unexpected and unusual but important metabolites. Moreover, the metabolomics approach has advantages over methods using radiolabeled drugs: The metabolomics approach is more environmentally friendly as radiolabeled compounds are not needed, and metabolites lacking a radiolabeled center can be uncovered.

Additionally, the LC-MS-based metabolomics approach is especially effective in screening reactive drug metabolites. Reactive metabolites are considered as important components that cause drug-related toxicity. The bioactivation of parent compounds can be mediated by different enzymes, including cytochrome P450 (CYP) enzymes, peroxidases, amine oxidase, and flavin-containing monooxygenases (FMO) (Tang and Lu 2010). The unstable intermediates, such as α,β-unsaturated carbonyls, epoxides, isothiocyanates, quinones, quinone imines, and quinone methides, are highly reactive. These reactive metabolites may react with macromolecules in cells like protein and DNA (Tang and Lu 2010; MacKenzie et al. 2020) to form covalent bonds at important amino acid residues, which may lead to the deactivation of enzymes and cause toxicity. Similarly, the binding of bioactive metabolites to DNA molecules will result in the instability of the genome and genotoxicity (Tang and Lu 2010; Pal et al. 2021). The untargeted LC-MS-based metabolomics approach is capable of discovering these drug-related adduct peaks and identifying the putative structures for these metabolites (Li et al. 2011, Li et al. 2014). The information concerning stable and reactive metabolites will provide some clues for the roles of drug metabolism in its toxicity. It is worth noting that the absolute quantification of metabolites could not be achieved when standards of metabolites are not available, employing an LC-MS-metabolomics approach. Thus, it is challenging to establish a metabolite concentration-toxicity relationship.

Identifying Biomarker and Mechanisms of Drug Toxicity

The LC-MS-based metabolomics approach plays important roles in discovering potential biomarkers for disease diagnosis and therapy outcome evaluation, as well as biomarker identification related to drug toxicity. For metabolomics, the extrapolation of cell culture profiling results to whole systems, or the extrapolation of one animal species to another is much easier compared with other omics approaches, as the chemical nature of certain metabolites remains the same across different systems and species (Balcerczyk et al. 2020). The LC-MS-based metabolomics approach can offer metabolome data for evaluating abnormal metabolism caused by toxicant administration, which is the cornerstone in further exploring toxicity mechanism. The optimal strategy for drug toxicity biomarker discovery and identification is decided by multiple factors like the scientific question, the choice of analytical platforms and databases, and the biological sample type, which have been thoroughly discussed above (Mastrangelo et al. 2015; Dunn et al. 2011; Dudzik et al. 2018). The receiver operating characteristic (ROC) curve, a nonparametric analytical method, is often adopted to evaluate the specificity and sensitivity of a biomarker. An area under the curve larger than 0.7 (AUC >0.7) is the minimum criterion for the performance of a biomarker test to be considered clinically useful (Alonso et al. 2015).

Investigating mechanisms of drug toxicity remains challenging. Until now, few mechanisms of drugs have been clarified. For one, drug combinations are often used in clinical settings, making it hard to distinguish the perpetrator of toxicity. Another consideration is that the species and individual differences further complicate the mechanistic studies of drug toxicity. Additionally, a clinically relevant animal model of drug toxicity is still not available in many cases (Lu et al. 2019). The metabolomics approach contributes significantly to answering remaining questions as evidenced by its growing applications in both preclinical and clinical studies. Novel mechanisms associated with drug toxicity have been revealed by building on metabolomics analysis and transgenic animal models (Li et al. 2014). Recently, scientists have begun to associate evidence from drug-mediated toxic phenotypes, discoveries from other omics approaches, and molecular biological experiments with aberrant metabolic pathways discovered in metabolomics analysis to determine the mechanisms of action in drug injury. For example, Jiang et al. combined proteomics and metabolomics approaches to study the long-term side effects of tramadol, an antituberculosis drug (Jiang et al. 2021). Proteomics analysis revealed 31 differentially expressed proteins in cardiac blood samples, while 29 significantly perturbed metabolites were identified from the metabolomics experiment. From these discoveries, it was concluded that the upregulation of isoleucine, valine, leucine, phenylalanine, and glutamine and the downregulation of carboxypeptidase B2 (CPB2) were the mechanisms of tramadol-induced toxicity by authors (Jiang et al. 2021). Zhang et al. studied the effects of benzene on AHH-1 (human peripheral blood B lymphocyte) cell metabolism and a benzene-exposed population (Zhang et al. 2021). Untargeted metabolomics first revealed changes in arginine biosynthesis, citrate cycle, glycine, serine, and threonine metabolism pathways in the experimentally treated cells and in blood samples from a benzene-exposed population. Targeted metabolomics, emphasizing the amino acid metabolism pathway, was subsequently conducted to verify the increased ratio of sarcosine/glycine. With the additional use of an mRNA array assay, it was discovered that the transcriptional level of glycine N-methyltransferase (GNMT), an enzyme catalyzing the transformation of glycine to sarcosine, was upregulated both in benzene-treated AHH-1 cells and in benzene-exposed workers. The findings indicated that the glycine/GNMT/sarcosine system was involved in benzene-induced hematotoxicity (Zhang et al. 2021). From the findings of a myriad of different studies, the conclusion is that drug toxicity mechanisms cannot be easily postulated from metabolomics data alone, which present more of a correlation than a strict causal relationship. However, the utilization of untargeted and targeted metabolomics approaches contributes enormously to the hypothesis generation and validation for the exploration into unknown mechanisms of drug-mediated toxicity.

Drug-Induced Hepatotoxicity

Drug-induced liver injury (DILI) represents one of the most cited toxicities associated with medications, accounting for around 50% of clinical cases of acute liver

failure and most cases of drug withdrawal from the market (Lu et al. 2019). The liver is the major organ responsible for the drug metabolism, which is considered to play an important role in DILI. Deeply understanding of the mechanism underlying DILI facilitates us to predict, prevent, and manage toxicity (Lu et al. 2019). As already discussed in section "Screening Drug Metabolites," the covalent modifying of metabolic enzymes and other proteins is considered one potential mechanism for DILI (Tang and Lu 2010, Pal et al. 2021). The depletion of the in vivo thiol pool (e.g., glutathione), such as in cases of acetaminophen overdose, significantly increases the reactive oxygen species (ROS) level, which will result in irreversible mitochondrial impairment and the death of hepatocytes (Tang and Lu 2010). Various biological systems for DILI studies can be employed for LC-MS-based metabolomics, such as primary hepatocytes and liver tissues from drug-treated animals. For example, the clinical cases of liver injury caused by saquinavir, an HIV protease inhibitor, have been reported (Sulkowski 2004). Therefore, researchers used an LC-MS-based metabolomics approach to profile the metabolism of saquinavir in human liver microsomes (HLM) and mice. Twenty novel metabolites were discovered including a reactive metabolite aldehyde. CYP3A4 was identified as the major enzyme responsible for saquinavir metabolism and bioactivation. CYP3A4-mediated α-hydroxyaldehyde formation may contribute to saquinavir-induced liver toxicity (Li et al. 2014). Aflatoxin B1 (AFB1) exposure has been well recognized as a risk factor for hepatocellular carcinoma (HCC). Researchers utilized the Hep3B cell line and LC-MS-based untargeted metabolomics to study the systemic metabolic reprogramming after AFB1 exposure. The analysis revealed 392 differential metabolites and discovered that there is an association of altered purine and pyrimidine metabolism, a decrease of hexosamine pathways, and sialylation with AFB1 exposure. These metabolites can probably serve as the biomarkers for AFB1 toxicity and the pathogenesis of AFB1-induced HCC (Wang et al. 2021b).

Drug-Induced Toxicity in Other Organs

Many chemotherapeutic drugs cause organ-specific adverse effects. LC-MS-based metabolomics are also applied to biomarker identification or mechanistic studies of drug-induced cardiac toxicity and acute kidney injury, as represented by the two following cases. Serum enzyme levels of aspartate transaminase (AST) and creatine phosphokinase (CPK) are commonly used as indicators of cardiac function, but AST and CPK are relatively insensitive. Combining these biomarkers with small metabolites identified from metabolomics may provide a better solution for the early diagnosis of cardiac toxicity and distinction of phenotypes. Alhazzani et al. examined whether the co-administration of valsartan is able to alleviate the cardiotoxic effects caused by doxorubicin in rats (Alhazzani et al. 2021). Doxorubicin treatment causes the decrease of some amino acids, such as alanine, valine, aspartic acid, and proline, as analyzed by serum metabolomics. These changes were rescued by the co-administration of valsartan. Additionally, the combination group revealed lower serum AST and CPK levels, a lower percentage of fibrosis, and less inflammation by

hematoxylin-eosin staining when compared with doxorubicin administration alone (Alhazzani et al. 2021).

Cisplatin, an antineoplastic drug, causes acute kidney injury. Tan et al. utilized a targeted metabolomics approach to focus on the disturbance of tryptophan pathway in rats treated with different doses of cisplatin and the protective effects of chlormethiazole, a CYP2E1 inhibitor (Tan et al. 2021). Blood urea nitrogen (BUN) and serum creatinine (Scr) were used to indicate the renal function. The metabolites related with the tryptophan pathway were quantified, and 16 metabolites were found to have been significantly altered after cisplatin treatment. Twelve of the sixteen metabolites were either positively or negatively correlated to cisplatin doses as expected. The renal expression of the key metabolic enzymes in the tryptophan pathway examined with Western blotting suggested that the change of tryptophan metabolism after cisplatin treatment is in line with the trend of altered expression in the corresponding metabolic enzyme. This cisplatin-induced kidney toxicity study is a good example of elucidating biomarker identities by linking metabolomics analysis with biological experiments (Tan et al. 2021). Moreover, using LC-MS-based metabolomics together with indicators of organ function is an effective way to study organ-specific toxicity.

Discussion and Future Directions of Biomarker Identification

This chapter briefly discussed different platforms used in metabolomics studies by touching upon several well-documented studies regarding metabolomics (Alseekh et al. 2021; Dunn et al. 2011; Want et al. 2013; Balcerczyk et al. 2020). This is a good time to focus on LC-MS-based metabolomics in the mechanistic study and biomarker identification of drug toxicity with well-established workflow. However, currently, LC-MS-based metabolomics is facing multiple challenges in this task. Sample preparation method optimization is extremely difficult for untargeted LC-MS-based metabolomics for multiple reasons. One such issue is that metabolomics studies require sample preparation covering a multitude of possible metabolites. Meanwhile, LC-MS prefers the use of clean samples with less biomatrix in order to improve sensitivity and resolution. Thus, method selection frequently has to make compromises on preferences based on the purpose of study. Though databases for metabolomes have been significantly improved and a number of software were developed for metabolite identification, many unknowns remain. But with the advance of isolation techniques and the reference from NMR technology, the issue of metabolite identification may be solved in the future. Numerous biomarker identification studies of drug toxicity are performed in cell lines and tissues from animal models using metabolomics strategies. However, the clinical relevance of identified biomarkers from animal models is concerning. Currently, more and more metabolomic-based biomarker identification is conducted in human samples, which could address this concern. As discussed in section "Experiment Design," a large-scale study of human samples is needed in order to determine a valid biomarker with

high accuracy, sensitivity, and specificity based on metabolomics as huge variations exist in human samples.

Newly emerging mass spectrometry imaging (MSI) technologies enable the spatial mapping of metabolites on cryo-sectioned tissue slides, which could be a promising approach for in situ biomarker identification of drug toxicity if paired with normal LC-MS metabolomics (Tong et al. 2021). The integration of diverse omics data by using genomics, transcriptomics, and proteomics in parallel has achieved great progress in biomarker identification. A strategy utilizing a multi-omics approach could be a promising future direction for biomarker identification by combining genetic aberrations, epigenetic alterations, changes in gene transcription, signaling pathways, and metabolic alterations (Gomez-Cebrian et al. 2021). For example, the concept of "metabolite genome-wide association studies (mGWAS)" has been claimed as the joining of metabolomics and genomics data, allowing the identification of a large number of genomic variations associated with metabolite levels. Nonetheless, the multi-omics approach still confront many challenges. To fulfill the purpose of reconstructing a reliable biochemical multi- or trans-omics network, highly integrated data with throughput and improved comprehension are needed before considering this kind of work as a regular approach in molecular biological studies and drug development (Gomez-Cebrian et al. 2021).

Put into greater context, a great deal of effort has been made to determine the reliable biomarkers of drug toxicity to improve drug safety. LC-MS-based metabolomics provides an efficient approach to determine the mechanisms or biomarkers of drug toxicity from metabolic pathways at the molecular level. The LC-MS-based metabolomics approach has also shown great strength in profiling drug metabolism and elucidating the mechanism of drug toxicity. Given its sensitivity, selectivity, speed, and robust nature, LC-MS-based metabolomics has been and will continue to be a powerful tool for identifying biomarkers and mechanisms associated with drug toxicity.

Applications to Other Diseases or Conditions

LC-MS-based metabolomics can be applied to the discovery and identification of biomarkers for drug toxicity as well as for other diseases like cancers, metabolic diseases, and neurodegenerative disorders. The identification of biomarkers for other diseases follows a similar workflow and utilizes the same databases as the drug toxicity biomarker identification.

Mini-dictionary of Terms

- **Gas chromatography (GC)-mass spectrometry (MS)** is an analytical method that combines the gas chromatography and mass spectrometry to analyze biological samples.

- **^{1}H-nuclear magnetic resonance (NMR) spectroscopy** is the application of NMR measuring the chemical shift of natural hydrogen (^{1}H) within a molecule and generates the signal sum of all the analytes containing ^{1}H.
- **Liquid chromatography (LC)** is a separation and enrichment technique that utilizes the discrepancy of analyte retention by stationary phase and elution by liquid mobile phase to separate a sample into individual parts.
- **LC-MS** is a powerful tool to analyze biological samples, which combines liquid chromatography (LC) with mass spectrometry (MS).
- **Metabolome** is the collective name for small biological molecules that present within an organism, cell, or tissue with molecular weights less than 1.5 kDa.
- **Metabolomics** is the systemic identification and quantification of the metabolome of an organism, cell, or tissue.
- **Metabolite genome-wide association studies (mGWAS)** is the joining of metabolomics and genomics data, allowing the identification of a large number of genomic variations associated with metabolite levels.
- **MS** is an analytical technique that identifies analytes with the mass-to-charge ratio of the ions.
- **Pharmacometabolomics**, stemming from metabolomics, is the comprehensive study of metabolites to evaluate pharmacokinetic profile (absorption, distribution, metabolism, excretion, and toxicity) of a drug and to map the drug effects on metabolism and certain endogenous metabolic pathways (pharmacodynamics).
- **Reactive metabolites** are unstable and highly reactive intermediates, such as α,β-unsaturated carbonyls, epoxides, quinones, quinone imines, and quinone methides, which could react with macromolecules (e.g., proteins and nucleic acids) and antioxidants (e.g., glutathione).

Key Facts of "LC-MS-Based Metabolomics in the Identification of Biomarkers Pertaining to Drug Toxicity: A New Narrative"

- Metabolomics is the systemic identification and quantification of small biological molecules, which has been successfully applied to the identification of biomarkers related to drug toxicity.
- Metabolomics is categorized into untargeted and targeted metabolomics, which are hypothesis generating and hypothesis-driven, respectively.
- LC-MS is the "workhorse" in the metabolomics study due to its high sensitivity, suitability for the detection of thermally labile nonvolatile metabolites, and large dynamic detection range from picomole to millimole.
- LC-MS-based metabolomics can not only monitor xenobiotics and their metabolites but also analyze endogenous metabolic pathways altered by drug medication.
- The workflow for untargeted LC-MS-based metabolomics approach comprises experiment design, sample collection and preparation, data acquisition, data preprocessing and analysis, metabolite identification, and data interpretation.

- The integration of data from diverse omics, including genomics, transcriptomics, and proteomics together with metabolomics in parallel, has achieved great progress in biomarker identification.

Summary Points

- LC-MS-based metabolomics is the identification and quantification of small molecules in an organism, cell, or tissue with LC-MS as the analytical method.
- Metabolomics is the closest to the phenotypes associated with drug toxicity, as the downstream of genomics, transcriptomics, and proteomics.
- Metabolomics approach shows advantages in easier extrapolation from preclinical to clinical compared with other omics like genomics, transcriptomics, and proteomics.
- LC-MS can be used in both untargeted metabolomics to generate the hypotheses concerning drug toxicity and targeted metabolomics to validate potential biomarkers.
- LC-MS-based metabolomics approach contributes to the identification of drug toxicity biomarker and the mechanistic elucidation of drug toxicity by screening drug metabolites and revealing changed endogenous metabolic pathways.
- LC-MS-based metabolomics is an ever-growing approach in biomarker discovery of drug toxicity due to its sensitivity, selectivity, and speed.

Cross-References

► Biomarkers of Lead Exposure: Platforms and Analysis
► Biomarkers of Liver Injury due to Toxic Agents: Progress, Current Applications, and Emerging Directions
► Drug-Induced Nephrotoxicity and Use of Biomarkers

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Bovine Whole Blood Cells as a Biomarker Platform for Biological Toxicology: A Focus on Thiacloprid

26

Martina Galdíková, Beáta Holečková, and Viera Schwarzbacherová

Contents

Abstract

In the present work, we deal with using bovine whole blood cells in cytotoxicity and genotoxicity studies of neonicotinoid insecticide thiacloprid (formulation and pure agent) in vitro. We chose bovine whole blood cells as potential bioindicators of environmental pollution, because ruminants are animals of the first contact with pollution via pasture. Related to thiacloprid-based insecticide formulation, comet assay and cytogenetic endpoints (chromosome aberrations, sister chromatid exchanges, and micronucleus test) showed the increase in the frequency of DNA damage, in unstable chromosomal aberrations (breaks) and sister chromatid exchanges in treated cell cultures. Significant reduction of cytochalasin-blocked proliferation index (CBPI) was determined suggesting the effect on the cell cycle delay (Galdíková et al., J Environ Sci Health B 50(10):698–707, 2015). Moreover, oxidant-based DNA damage, apoptosis, DNA damage response (e.g.,

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generation of micronuclei), and DNA double-strand breaks were documented in bovine lymphocytes (Schwarzbacherová et al., Toxicol In Vitro 61:104654, 2019). In the study of Galdíková et al. (Toxicol In Vitro 61:104616, 2019) that focused on the pure thiacloprid, replication and DNA damage and possible ability to induce cytotoxic/cytostatic effects and to influence the cell cycle were observed. The obtained results showed that bovine whole blood cells, mainly peripheral lymphocytes, are good bioindicators of the early effect of thiacloprid and pesticides in general. They might be used as one of the valuable steps in prognosis of possible negative effects of pesticides similarly as human peripheral lymphocytes. At present days it is known that chronic and sublethal pesticide exposure increases the risk of cancer but also other diseases or conditions such as asthma, diabetes, leukemia, Parkinson's and Alzheimer's disease, infertility, birth defects, autism, and others (Kim et al., Sci Total Environ 575:525–-535, 2017; Mostafalou and Abdollahi, Arch Toxicol 91:549–-599, 2017).

Keywords

Neonicotinoids · Thiacloprid · Bovine whole blood cells · Biomarker · Genotoxicity · Cytotoxicity · DNA damage · Chromosome aberrations · Micronuclei · Sister chromatid exchanges · Oxidative stress

Abbreviations

α4β2	The most prominent subtype of nicotinic acetylcholine receptors
BEAS-2B	The line of human bronchial epithelial cells
CaCo-2	The line of human intestinal cells
CAs	Chromosomal aberrations
CBPI	Cytokine-blocked proliferation index
CHOK1	Chinese hamster ovary cells
CMNN	Cytokinesis-blocked micronuclei assay
DSBs	Double-strand breaks
EPA	Environmental Protection Agency
FISH	Fluorescence in situ hybridization
GSTs	Glutathione S-transferases
HepG2	The line of human hepatocellular carcinoma cells
IMR-90	Lung fibroblast cells
MNi	Micronuclei
nAChRs	Nicotinic acetylcholine receptors
NO	Nitric oxide
PC12	The line of rat pheochromocytoma adrenal medulla cells
ROS	Reactive oxidative species
SCE	Sister chromatid exchanges
SH-SY5Y	The line of human neuroblastoma cells
THI	Thiacloprid
THI-NH	Descyanothiacloprid
THI-SO	Thiacloprid-sulfoxide

TK	Thymidine kinase
TK6	The line of human lymphoblastoid cells
V79	The line of Chinese hamster cells
WHO	World Health Organization

Introduction

Extensive agricultural production requires increased application of fertilizers and pesticides to protect crops from harmful insects, fungal diseases, and weeds. Alongside their beneficial effects, there are also many risks associated with pesticides, in particular their toxicity to nontarget organisms. In addition to acute poisoning caused, for example, by accidental ingestion, chronic exposure to low doses is also dangerous and can lead to the development of various diseases such as Parkinson's disease (Van Maele-Fabry et al. 2012; Islam et al. 2021), Alzheimer's disease (Yan et al. 2016; Tang 2020), asthma (Buralli et al. 2020), diabetes (Velmurugan et al. 2017; Park et al. 2019), reproductive problems (García et al. 2017; Fucic et al. 2021), and cancer (Alavanja and Bonner 2012; Parrón et al. 2014). Some of these substances have been considered potential genotoxic, and the effects on the genetic material, in particular, are among the most dangerous side effects of pesticides. They can directly induce structural or functional damage of chromosomes, DNA, and histone proteins or indirectly affect gene expression by altering cell organelles or other factors which are involved in maintaining cell integrity (Mostafalou and Abdollahi 2013; Kaur and Kaur 2018).

Animals come into contact with chemical substances such as pesticides, especially through the feed. Grazing animals in particular, which consume huge quantities of contaminated feed, are a good model for monitoring the undesirable effects of pesticides (Rhind 2005). Considering that cows are a component of the human food chain, there are many concerns regarding the accumulation of chemical substances such as pesticides in cattle tissues and milk (Seccia et al. 2008; Pardío et al. 2012).

Neonicotinoid Insecticides

Neonicotinoids are the newest group of pesticides that have been used broadly in agriculture and veterinary medicine against pests. These systemic insecticides act as nicotinic acetylcholine receptor (nAChR) agonists in the central nervous system of insects, and their presumed low toxicity to mammals, together with their wide-ranging insecticidal activity and unique mode of neurotoxic action, contributes to their popularity (Tomizawa and Casida 2005; Li and Kannan 2020). However, properties such as good water solubility and long persistence and accumulation in environment are associated with undesirable effects on nontarget insects, in particular honey bees and bumble bees (Alkassab and Kirchner 2018; Christen et al. 2018; Havstad et al. 2019), aquatic invertebrate and vertebrate species (Malhotra et al.

2021), birds (Hallmann et al. 2014; Li et al. 2020), and mammals (Berheim et al. 2019; Costas-Ferreira and Faro 2021). In relation to undesirable effects on bees, the EU banned in 2018 imidacloprid, clothianidin, and thiamethoxam.

Neonicotinoids have been found to affect nAChR in mammals in a similar manner to the effects of nicotine (Kimura-Kuroda et al. 2012). Most mammalian adverse toxic effects are connected with their action on binding to the α4β2, the most prominent subtype of nAChRs in mammals, with the highest density of receptors in the thalamus (Li et al. 2011). Other studies have revealed reproductive toxicity as well as harmful effect on embryonic development (Gu et al. 2013; Mikolić and Brčić Karačonji 2018). The teratogenic effect was described in rat fetuses after exposure to imidacloprid (Gawade et al. 2013) and in chick embryos (Hussein et al. 2015). Authors Babeľová et al. (2017) demonstrated adverse effect of four neonicotinoids on preimplantation embryos in mice and rabbits. In addition to the above effects, many studies have shown the endocrine-disrupting potential of neonicotinoids (Kapoor et al. 2011; Caron-Beaudoin et al. 2018). Authors Şekeroğlu et al. (2014) observed statistically increased levels of free triiodothyronine 3 and free thyroxine hormones in serum of rats after treatments with thiacloprid and deltamethrin. In another study, thiacloprid, imidacloprid, and thiamethoxam increased the production of estradiol and estrone and inhibited the production of estriol (Caron-Beaudoin et al. 2017).

It is well documented that neonicotinoids are also associated with oxidative stress. According to Wang et al. (2018), oxidative stress may be the most common toxicological mechanism of neonicotinoids. Thus, formation of lipid, protein, and DNA damage is due to the generation of reactive oxidative species (ROS) and decreased capacity of antioxidant defense (Mostafalou and Abdollahi 2013). Authors Yan et al. (2020) demonstrated that exposure to nitenpyram, acetamiprid, and dinotefuran induced oxidative stress in the liver of mice. All treatment groups showed decreased levels of free radical scavenger glutathione (GSH). In addition, exposure to insecticides led to the accumulation of lipids in the liver. Similarly, authors Hendawi et al. (2016) observed an increase in lipid peroxidation together with simultaneous statistically significant decrease in Phase II detoxification enzymes glutathione S-transferases (GSTs) in rats after the exposure to thiacloprid.

Thiacloprid

Thiacloprid [3-(6-chloro-3-pyridinylmethyl)-2-thiazolidinylidene]cyanamide is a chloronicotinyl insecticide widely used as an active compound in commercial formulation Calypso® 480 SC against sucking and chewing pests. Thiacloprid (THI) is a systemic neonicotinoid that is taken up by the plant roots or leaves and is translocated to all parts of the plants, which makes them toxic to insects. In mammals, THI is rapidly absorbed and, after subsequent metabolic processes, rapidly excreted, with little remaining in the tissues. Thiacloprid metabolization involves five different sites of attack: cyano hydrolysis, sulfoxidation, hydroxylation at the 4-position, conversion to olefin, and loss of the cyano group. The urea

derivative (THI-4-OHNCONH2) and THI-SO are the major metabolites observed (Ford and Casida 2008). The action of cytochromes P 450 on thiacloprid in vivo generates descyanothiacloprid (THI-NH). This step is considered as bioactivation of thiacloprid, because THI-NH is toxic to mammals due to its potential binding to α4β2 nicotinic acetylcholine receptors (Chao and Casida 1997); on the contrary, in insects, THI-NH serves as a metabolite of detoxification (Simon-Delso et al. 2015). As demonstrated by Aydin (2011), acute and subacute exposure of rats to thiacloprid caused a decrease in the level of antioxidant enzymes in all lymphoid organs and the plasma. This decrease led to oxidative stress caused by elevated nitric oxide (NO) levels, which increased myeloperoxidase activity or lipid peroxidation. Subsequently, the increase in nitric oxide activity may be associated with the higher production of peroxynitrite and hydroxyl radicals, oxidative stress, DNA damage, and/or apoptosis. Today it is well known that the primary target organ of thiacloprid is the liver, similarly to other pesticides. In a study by Alarcan et al. (2020), hepatotoxic effect of thiacloprid, imazalil, and clothianidin was confirmed. The authors observed changes in the liver of female Wistar rats after 28 days' exposure to single pesticide or their mixture. Based on thyroid tumors and uterine tumors in rats and ovary tumors in mice, thiacloprid has been classified as a "likely" human carcinogen (EPA 2013) or moderately hazardous (WHO 2009).

Genotoxicity of Thiacloprid

In mutagenicity tests, no positive results have been obtained in bacterial assays using *Salmonella typhimurium* or *Escherichia coli*. Similarly, no clastogenic effects were observed in in vivo assays for induction of micronuclei in mice or in chromosome aberration test in Chinese hamster V79 cells (Pesticide residues in food 2006). On the contrary, several studies have shown potential genotoxic effects of thiacloprid on human and animal health. Authors Calderón-Segura et al. (2012) recorded the induction of DNA damage in a concentration-dependent manner in human peripheral lymphocytes after exposure to neonicotinoid commercial formulations containing thiacloprid, imidacloprid, and clothianidin. In the in vivo study by Şekeroğlu et al. (2013), rats were treated orally with different doses of thiacloprid and deltamethrin alone or in mixture. The results showed significant increases in chromosomal aberrations (CAs) and micronuclei (MNi) frequencies after all treatments in bone marrow cells of rats. Similarly, increased formation of CAs, MNi, and sister chromatid exchanges (SCEs) in human lymphocytes was obtained after exposure to different concentrations (75, 150, and 300 μg ml^{-1}) of thiacloprid. Decreases in mitotic, nuclear division, and proliferation indices at all concentrations were also reported (Kocaman et al. 2014). Şenyildiz et al. (2018) documented genotoxic and cytotoxic effects of thiacloprid, thiamethoxam, acetamiprid, clothianidin, and imidacloprid in human neuroblastoma (SH-SY5Y) and human hepatocellular carcinoma (HepG2) cells. They found thiacloprid being most genotoxic in SH-SY5Y cells as well as most cytotoxic in both cell lines. Recently, Şekeroğlu et al. (2021) reported decreased cell viability and colony formation in human bronchial epithelial

cells (BEAS-2B) after exposure to thiacloprid and deltamethrin. Treatment for 120 h also showed statistically significant induction of γH2AX and 53BP1 foci formation at the highest concentration (44 μM deltamethrin + 666 μM thiacloprid). Using biophysical methods, the effect of thiacloprid on the structure and stability of calf thymus DNA has been studied by Verebová et al. (2019) in the recent past. The authors concluded that thiacloprid changes the structure and stability of calf thymus DNA via binding into the minor groove by hydrophobic or hydrogen interactions.

Genotoxicity of Other Neonicotinoids

When compared with thiacloprid, similar genotoxic potential of another member of neonicotinoid insecticides – acetamiprid – was confirmed in human peripheral lymphocytes. Acetamiprid significantly increased frequencies of CAs, SCEs, and MNi (Kocaman and Topaktaş 2007). Positive results were documented by Çavaş et al. (2012) after exposure of human intestinal CaCo-2 cells to acetamiprid using the micronucleus, comet, and γH2AX foci formation assays. In another study, acetamiprid induced generation of micronuclei and DNA damage measured using alkaline comet and γH2AX foci formation assays in human lung fibroblast cells (IMR-90) (Çavaş et al. 2014). Similarly, in a study performed by Annabi et al. (2019), acetamiprid showed genotoxic and cytotoxic effect on rat pheochromocytoma adrenal medulla (PC12) cells. As concerns clothianidin, its genotoxic and cytotoxic effect was confirmed in human peripheral lymphocytes with or without metabolic activation system using cytogenetic endpoints (Atlı Şekeroğlu et al. 2019). Clothianidin also induced oxidative stress and formation of DNA breaks measured using comet test and γH2AX assay in bronchial epithelial cells (Atlı Şekeroğlu et al. 2020). The genotoxic effect of imidacloprid has also been evaluated by several researchers. Feng et al. (2005) investigated the genotoxic effects of imidacloprid using comet assay and cytogenetic endpoints in human peripheral blood cells and reported significantly increased DNA damage in all treatment groups, whereas SCE and MNi frequencies were observed only in higher concentrations. Similar results were observed by Costa et al. (2009) in human lymphocytes. They found a significant increase in comet assay and MNi formation only in the highest tested concentration of imidacloprid. Furthermore, genotoxicity of imidacloprid on the Chinese hamster ovary cells (CHOK1) revealed observed significant induction of micronuclei, however no elevation in chromosome or DNA damage (Al-Sarar et al. 2015). In Guo et al.'s study, thymidine kinase (TK) gene mutation assay, the comet assay, and the micronucleus test were employed for the assessment of genotoxic effect of imidacloprid in human lymphoblastoid TK6 cells. Authors found elevation in the micronucleus (MN) frequency, TK mutations, and DNA damage in a dose-dependent manner without increase of ROS (Guo et al. 2020). Conversely, authors Guimarães et al. (2021) observed the cytotoxicity of imidacloprid HepG2 cells associated with an increase in the generation of reactive oxygen and nitrogen species.

Bovine Cell Cultures

Blood cells are among the most common cell types used in genotoxicity studies. They have some advantages: they are easily accessible cells, and as they circulate through the whole body, they may mimic effects occurring in remote tissues.

Cytogenetic studies in peripheral lymphocytes have been employed as a biological dosimeter to estimate the effect of genotoxic agents on farm animals. The study of the genotoxicity of environmental mutagens in domestic animals is of particular concern because of their possible consequences on the productive and reproductive efficiency of livestock. Moreover, dairy cattle are an important link in the food chain, and the accumulation of xenobiotics in meat and milk should be regarded as hazardous to human health (Rubeš et al. 1997; Lioi et al. 1998).

Bovine whole blood cells have been commonly used as a valuable tool for the evaluation of genotoxic effects induced by many different chemicals and toxic substances present in the environment (Šiviková et al. 2013, 2018; Rossi et al. 2014; Drážovská et al. 2016; Schwarzbacherová et al. 2017; Ferré et al. 2020).

Authors Galdíková et al. (2015) studied the potential genotoxic effect of commercial formulation of thiacloprid (trade name Calypso® 480 SC) on bovine peripheral lymphocytes in vitro. Cell cultures were treated with thiacloprid-based insecticide at concentrations 30, 60, 120, 240, and 480 μg ml^{-1}. Statistically increased frequency of DNA along with chromosomal damage was observed after the exposure to insecticide at concentrations ranging from 120 to 480 μg ml^{-1}. Stable structural and numerical aberrations were observed without statistical significance using fluorescence in situ hybridization technique (FISH). Authors also found dose-dependent elevation in SCE induction after 48 h exposure. In addition, reduction of mitotic, proliferation, and cytochalasin-blocked proliferation indices was recorded. On the basis of their results, the authors assumed that prolonged incubation (48 h) with thiacloprid formulation might result in the inhibition of DNA synthesis and cell proliferation.

In the study by Koleničová et al. (2019), alkaline and neutral comet assays were used for the assessment of the potential genotoxic effects of thiacloprid-based formulation and pure thiacloprid on isolated bovine lymphocytes after exposure for 2 h with and without pre-cultivation. By using the alkaline comet variant, the results showed an increase in DNA damage after exposure to Calypso® in the concentration range from 60 to 480 μg ml^{-1} and in pre-cultivated lymphocytes at the highest tested concentrations. Neutral comet assay resulted in statistically significant DNA damage only at the highest concentration. As regards the pure substance thiacloprid, an increased percentage of DNA damage was observed only at the highest concentration tested in the alkaline comet assay. DNA damage results detected using neutral comet test after exposure to thiacloprid did not confirm the genotoxic effect. By comparing different experimental conditions, the authors verified the suitability of the comet assay in assessing the possible effect on genetic material after the exposure to thiacloprid on bovine lymphocytes. Similarly, increased levels of percentage of comets were described by Galdíková et al.

(2015) in bovine lymphocytes, and the same was determined in human peripheral lymphocytes (Calderón-Segura et al. 2012).

Positive genotoxic and cytotoxic effects of thiacloprid were documented by Galdíková et al. (2019) after exposure of bovine blood cells to thiacloprid at concentrations of 30, 60, 120, 240, and 480 μg ml^{-1}. This study was a follow-up to their previous work (Galdíková et al. 2015), in which they tested the effect of a commercial thiacloprid-based formulation on bovine cells at the same concentrations. Using comet assay, significant DNA damage was observed at the highest concentrations (240 and 480 μg ml^{-1}) (Table 1). No statistical values were seen in the induced CAs after 24 and 48 h, except at the concentration of 120 μg ml^{-1}. The authors have chosen the lowest concentration of insecticide for FISH; however, using painting probes for chromosomes 1, 5, and 7, no statistically significant translocations or numerical aberrations (aneuploidies and polyploidies) were detected. Authors further observed a statistically significant increase in frequency of SCE after 24 h exposure to insecticide at concentrations from 120 to 480 μg ml^{-1} (Table 2). The tested insecticide failed to produce any statistical changes in MNi frequency after neither 24 nor 48 h treatment (Table 3). Additionally, statistically significant decreases in mitotic and proliferation indices were detected (Tables 2 and 3); therefore, researchers suggested that thiacloprid was found to be capable of inducing cytostatic and cytotoxic effects in cultured bovine whole cells.

Table 1 Percentage of damaged DNA and viability after 2 h exposure to active agent thiacloprid on non-proliferating bovine lymphocytes of peripheral blood

Dose	Viability (%)	DNA damage (% DNA in tail)
	Donor 1	
Control	95.0	3.20 ± 1.34
Thiacloprid (μg ml^{-1})		
60	93.8	4.18 ± 1.20
120	94.1	3.97 ± 1.29
240	94.7	5.18 ± 1.24
480	94.4	8.24 ± 0.83**
H_2O_2 (300 μg ml^2)	–	17.80 ± 2.22***
	Donor 2	
Control	98.9	2.85 ± 0.53
Thiacloprid (μg ml^{-1})		
60	97.7	3.45 ± 1.42
120	97.0	3.73 ± 0.91
240	97.0	5.83 ± 1.65*
480	96.7	5.69 ± 0.60**
H_2O_2 (300 μg ml^2)	–	17.46 ± 1.66***

DNA damage (% DNA in tail) is presented as a mean value ± standard deviation; a total of 100 cells of each donor and concentration were analyzed, H_2O_2 (hydrogen peroxide) used as positive control; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ – statistical significance using Student's t-test. (From Galdíková et al. (2019) with permission)

Table 2 Frequency of SCEs and proliferation indices in cultured peripheral lymphocytes exposed to thiacloprid for 24 and 48 h

Dose	SCE/cell ± SD	PI
Donor 1		
Control	5.00 ± 2.27	2.21
Thiacloprid (g ml^{-1}) 24 h		
30	5.12 ± 1.60	2.13
60	5.60 ± 1.84	2.00**
120	6.26 ± 2.13**	1.98***
240	6.58 ± 2.37**	2.26
480	6.20 ± 2.00**	2.10*
0.4 M MMC	9.86 ± 2.30***	2.37
Donor 2		
Control	5.18 ± 1.74	2.12
Thiacloprid (g ml^{-1}) 24 h		
30	5.20 ± 1.47	2.10
60	5.75 ± 2.13	1.93**
120	6.36 ± 2.71**	1.90***
240	6.63 ± 2.49**	2.09
480	6.14 ± 2.35*	2.02*
0.4 M MMC	9.74 ± 3.03***	2.24
Donor 1		
Control	5.20 ± 2.30	2.22
Thiacloprid (g ml^{-1}) 48 h		
30	5.20 ± 1.66	2.20
60	5.28 ± 2.12	2.15
120	5.48 ± 1.89	2.11***
240	5.61 ± 2.39	2.01***
480	ND[a]	1.50***
0.4 M MMC	24.18 ± 6.24***	1.57***
Donor 2		
Control	5.36 ± 1.91	2.28
Thiacloprid (g ml^{-1}) 48 h		
30	5.36 ± 1.91	2.28
60	5.54 ± 1.79	2.26
120	5.58 ± 2.40	2.24***
240	5.65 ± 2.17	2.12***
480	ND[a]	1.65***
0.4 M MMC	27.08 ± 7.42***	1.55***

A total of 50 division metaphases of each donor and concentration were analyzed for SCE, and 100 metaphases were detected for the PI. SCEs/cell are expressed as mean ± SD; *PI* proliferation index, *MMC* mitomycin C used as a positive control. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ – statistical significant data; *ND* not done. ANOVA/Student's t-test was used for SCE and χ^2 test for the PI. (From Galdíková et al. (2019) with permission)

[a]Insufficient number of cells

Table 3 Induction of MN in bovine peripheral lymphocytes exposed to insecticide thiacloprid for 24 and 48 h

	Donor 1				**Donor 2**			
	BNMN		**% BNMN ± SD**	**CBPI ± SD**	**BNMN**		**% BNMN ± SD**	**CBPI ± SD**
	1	**2**			**1**	**2**		
Control	12	1	0.75 ± 0.07	1.225 ± 0.019	11	0	0.55 ± 0.07	1.238 ± 0.009
Thiacloprid (μg ml^{-1})								
30	9	1	0.55 ± 0.07	1.207 ± 0.032	11	1	0.65 ± 0.07	1.213 ± 0.007*
60	12	2	0.80 ± 0.14	1.179 ± 0.017*	15	2	0.95 ± 0.21	1.187 ± 0.008***
120	13	2	0.85 ± 0.07	1.167 ± 0.012	15	1	0.85 ± 0.07	1.162 ± 0.011***
240	14	0	0.70 ± 0.14	1.146 ± 0.007***	15	2	0.95 ± 0.21	1.153 ± 0.005***
480	14	1	0.80 ± 0.14	1.133 ± 0.021	12	1	0.70 ± 0.14	1.115 ± 0.016***
MMC	16	2	1.00 ± 0.14	1.11 ± 0.021***	18	2	1.10 ± 0.14*	1.124 ± 0.019***
	Donor 1				**Donor 2**			
	BNMN		**%BNMN±SD**	**CBPI±SD**	**BNMN**		**%BNMN±SD**	**CBPI±SD**
	1	**2**			**1**	**2**		
Control	12	0	0.60 ± 0.00	1.169 ± 0.013	13	0	0.65 ± 0.07	1.172 ± 0.009
Thiacloprid (μg ml^{-1})								
30	13	0	0.65 ± 0.07	1.156 ± 0.017	13	1	0.75 ± 0.07	1.153 ± 0.090**
60	17	2	1.05 ± 0.21	1.129 ± 0.024**	16	2	1.00 ± 0.14	1.131 ± 0.007***
120	16	2	1.00 ± 0.14	1.111 ± 0.010	19	1	1.05 ± 0.21	1.113 ± 0.008***
240	14	2	0.90 ± 0.14	1.107 ± 0.008***	15	2	0.90 ± 0.14	1.108 ± 0.007***
480	13	0	0.65 ± 0.21	1.098 ± 0.003	15	0	0.75 ± 0.07	1.096 ± 0.003***
MMC	34	2	1.9 ± 0.14***	1.11 ± 0.021***	36	3	2.1 ± 0.14***	1.124 ± 0.019***

For BNMN a total number of 2000 cells of each donor and concentration were analyzed and for CPBI 500 cells 4 times in row/concentration. All data are expressed as mean ± SD; *MN* micronucleus, *BNMN* binucleated cell with a micronucleus/micronuclei blocked by cytochalasin, *CBPI* cytokinesis-block proliferation index, *MMC* mitomycin C used as a positive control. Statistically significant data: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ANOVA, Student's test. (From Galdíková et al. (2019) with permission)

In the study conducted by Schwarzbacherová et al. (2019), oxidative stress, apoptosis, and genetic instability after in vitro short-term (4 h) exposure to different concentrations of thiacloprid-based formulation (10, 30, 60, 120, and 240 μg ml^{-1}) were confirmed. Authors found decreased viability and occurrence of apoptosis at concentrations ranging from 30 to 240 μg ml^{-1} besides p53-mediated cell cycle arrest at the G0/G1 phase. Further, significantly increased levels of mitochondrial superoxide and of carbonylated proteins confirming oxidative stress were detected (Fig. 1). Regarding genotoxicity, using CMNN (cytokinesis-blocked micronuclei assay), dose-dependent elevation of micronuclei together with decline in CBPI (cytokine-blocked proliferation index) was found (Fig. 2). Tested insecticide induces significantly higher levels of double-strand breaks (DSBs) at all tested concentrations in neutral comet assay (Fig. 3). The results of the authors contributed to the clarification of thiacloprid formulation effects on animal lymphocyte cultures after short-term exposure.

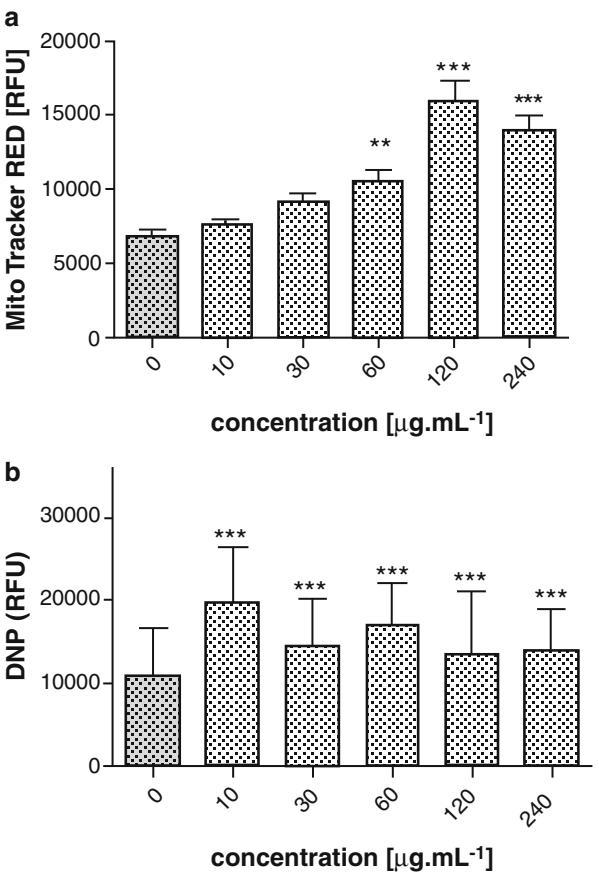

Fig. 1 Study of oxidative stress damage in bovine lymphocytes after thiacloprid formulation treatment. (**a**) MitoTracker® Red CM-H_2Xros was used to evaluate the mitochondrial superoxide levels. (**b**) Protein carbonylation was revealed by detecting the levels of DNP using rabbit anti-DNP antibody and goat anti-rabbit secondary antibody. Results are presented as relative fluorescence units (RFU). Applying to all results, the bars indicate SD, $n = 3$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to control (ANOVA and Dunnett's a posteriori test). (From Schwarzbacherová et al. (2019) with permission)

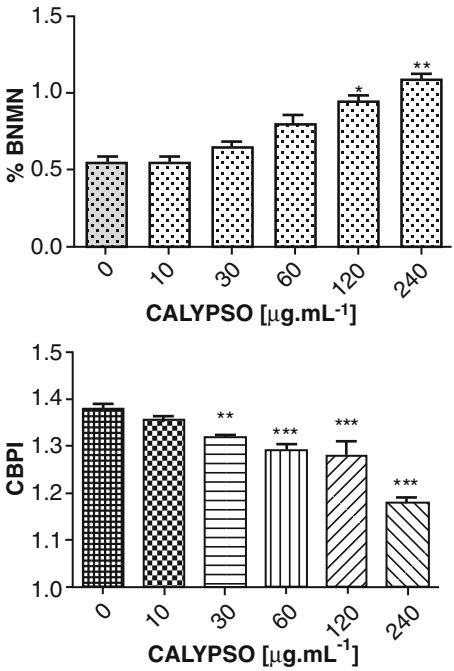

Fig. 2 Micronucleus induction and changes in cell proliferation treated with thiacloprid-based insecticide. CBMN assay was performed to evaluate the levels of micronuclei; a total of 1000 binucleated cells were scored. Changes in cell proliferation were assessed using the cytochalasin-blocked proliferation index (CBPI) after insecticide treatment. At least 500 cells were analyzed. Bars indicate SD, $n = 3$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared to control group (ANOVA and Dunnett's a posteriori test). (From Schwarzbacherová et al. (2019) with permission)

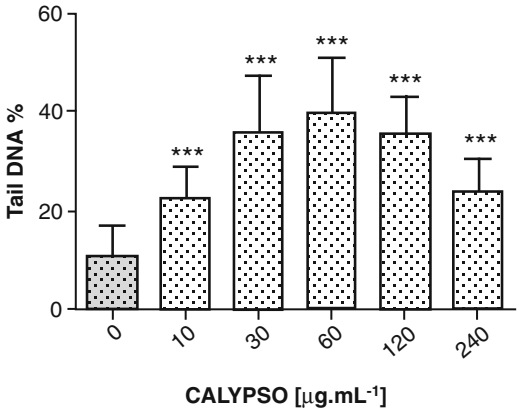

Fig. 3 Neutral comet assay analysis in bovine peripheral lymphocytes after thiacloprid-based insecticide. Incidence of DSBs (% tail DNA) was evaluated using neutral comet assay. The bars indicate SD, $n = 150$, $***p < 0.001$ compared to control (ANOVA and Dunnett's a posteriori test). The bars indicate SD, $n = 3$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared to control group (ANOVA and Dunnett's a posteriori test). (From Schwarzbacherová et al. (2019) with permission)

Applications to Prognosis, Other Diseases, or Conditions

In this study, we review using bovine whole blood cells in cytotoxicity and genotoxicity studies of neonicotinoid insecticide thiacloprid (formulation and pure agent) in vitro. We chose bovine whole blood cells as potential bioindicators of environmental pollution, because ruminants are animals of the first contact with pollution via pasture. Related to thiacloprid-based formulation, comet assay and cytogenetic endpoints showed an increase in the frequency of DNA damage, chromosomal aberrations (breaks), and sister chromatid exchanges. Reduction of cytochalasin-blocked proliferation index (CBPI) was also determined suggesting the effect on the cell cycle delay (Galdíková et al. 2015). Moreover, oxidant-based DNA damage, apoptosis, DNA damage response (micronuclei), and DNA double-strand breaks were documented (Schwarzbacherová et al. 2019). Galdíková et al. (2019) focused on the pure thiacloprid; replication and DNA damage and possible ability to induce cytotoxic/cytostatic effects and to influence the cell cycle were observed. The obtained results showed that bovine whole blood cells are good bioindicators of the early effect of thiacloprid. They might be used as one of the valuable steps in the prognosis of possible negative effects of pesticides on humans such as cancer and also diseases such as asthma, Parkinson's and Alzheimer's disease, infertility, birth defects, and autism.

Mini-dictionary

Genotoxin: A genotoxin is a chemical or agent that can cause DNA or chromosomal damage.

Chromosomal aberrations: Chromosomal aberrations are changes in chromosome structure or number.

DNA damage: DNA damage is a change in the basic structure of DNA.

Mutations: Mutations are permanent hereditary changes: somatic mutations are inherited through mitosis; mutations in gametes are transmitted from parents to offspring.

Neonicotinoid insecticides (neonicotinoids): Neonicotinoids are systemic insecticides that act as nicotinic acetylcholine receptor (nAChR) agonists in the central nervous system of insects.

Nicotinic acetylcholine receptors (nAChRs): nAChRs are neurotransmitter receptors in the insect central nervous system that play a crucial role in insect physiology.

Thiacloprid: Thiacloprid is a systemic neonicotinoid insecticide that is translocated to all parts of the plants, which makes them toxic to insects such as sucking and chewing pests.

Key Facts

- Bovine whole blood cells are suitable for genotoxicity studies.
- Cytogenetic studies in animal peripheral lymphocytes can serve as a biological dosimeter.
- Genotoxicity of environmental mutagens is of particular concern.
- Neonicotinoid insecticides are widely used in agriculture and veterinary medicine.
- Thiacloprid is a systemic neonicotinoid translocated to all parts of the plants; they can be toxic to insects.
- The toxicity to humans and animals is an ongoing concern.

Summary Points

- Bovine whole blood cells have been commonly used as a valuable tool for the evaluation of genotoxic effects induced by many different chemicals and toxic substances present in the environment.
- In this chapter the potential genotoxic effects of commercial formulation of thiacloprid (trade name Calypso® 480 SC) and pure thiacloprid on bovine peripheral lymphocytes in vitro are reviewed.
- Thiacloprid-based insecticide formulation induced the increase in the frequency of DNA damage, unstable chromosomal aberrations (breaks), and sister chromatid exchanges in bovine cell cultures. A significant reduction of cytochalasin-blocked proliferation index (CBPI) was determined suggesting the effect on the cell cycle delay.
- In addition, oxidant-based DNA damage, apoptosis, DNA damage response (formation of micronuclei), and DNA double-strand breaks were documented in bovine whole blood cultures exposed to thiacloprid formulation.
- The pure thiacloprid caused replication and DNA damage and possible ability to induce cytotoxic/cytostatic effects and to influence the cell cycle.
- The results showed that bovine whole blood cells, mainly peripheral lymphocytes, are good bioindicators of the early effect of pesticides.

Cross-References

► Micronucleus Assay in Lymphocytes for Human Biomonitoring and Clinical Studies

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Blood-Brain Barrier Function as a Biomarker in Toxicology: Impact of Environmental Toxicants

27

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Abstract

The blood-brain barrier (BBB) is a semipermeable endothelial barrier that plays a vital role in protecting and maintaining proper function of the brain and the central nervous system (CNS). The BBB tightly regulates the transport of nutrients and ions and prevents entry of pathogens and toxic substances into the brain. Recently, environmental toxicants such as polycyclic aromatic hydrocarbons, dioxins, heavy metals, perfluoroalkyl substances, and airborne pollutants have been implicated in altering the permeability of the BBB, thus making BBB function a viable biomarker of neurotoxicity. To assess BBB function after toxicant exposure, researchers have used a variety of in vitro and ex vivo approaches, each of which comes with their own advantages and disadvantages. In this chapter, we provide a general overview of the BBB and a discussion of the evidence that supports BBB function as a biomarker of toxicity and disease. We also discuss the in vivo, ex vivo, and in vitro approaches that currently are available to measure BBB permeability.

Keywords

Blood-brain barrier · Central nervous system · Brain · Neurotoxicity · Toxicants · Pollutants · Heavy metals · Polycyclic aromatic hydrocarbons · Dioxins · Transendothelial electrical resistance · Neurovascular unit · Brain microvascular endothelial cells · Microfluidics · Neurodegenerative disease

List of Abbreviations

ABC	Adenosine triphosphate-binding cassette
AD	Alzheimer's disease
AhR	Aryl hydrocarbon receptor
AQP4	Aquaporin 4
ATP	Adenosine triphosphate
BPAEC	Bovine pulmonary artery endothelial cell
BaP	Benzo[a]pyrene
BBB	Blood-brain barrier
BCRP	Breast cancer resistance protein
BMEC	Brain microvascular endothelial cell
BSCB	Blood-spinal cord barrier
CNS	Central nervous system
DNA	Deoxyribonucleic acid
EC	Endothelial cell
ECIS	Electrical cell-substrate impedance sensing
GDNF	Glial cell line-derived neurotrophic factor
hBMEC	Human brain microvascular endothelial cell
HRP	Horseradish peroxidase

MRI	Magnetic resonance imaging
Mrp	Multidrug resistance-associated proteins
NVU	Neurovascular unit
PAHs	Polycyclic aromatic hydrocarbons
PET	Positron-emission tomography
PFOS	Perfluoroctanesulfonic acid
Pgp	P-glycoprotein
Pl3K	Phosphatidylinositol 3-kinase
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
TEER	Transendothelial electrical resistance
TJ	Tight junction
WMH	White matter hyperintensities

Introduction

In 1885, Paul Ehrlich, a German physician, discovered that trypan blue dye, when injected intravenously into the mouse, stained every tissue in the body except the brain and the spinal cord (Ehrlich 1885). This was the first documented evidence of a physical barrier at the interface of the central nervous system (CNS) and the circulation, preventing free movement of substances from the blood into the CNS. This barrier, now known as the blood-brain barrier (BBB) in the brain, and the blood-spinal cord barrier (BSCB) in the spinal cord, is formed primarily of specialized endothelial cells that line the blood vessels innervating and surrounding the CNS. In this chapter, we focus on the BBB. Although similar to the BBB, the BSCB has distinct morphology and functions that are beyond the scope of this chapter. Information about the BSCB can be found elsewhere (see Bartanusz et al. 2011).

With the advent of electron microscopy in the 1950s, scientists were able to ascertain the ultrastructural features of the BBB (Ribatti et al. 2006). However, its pivotal role in maintaining homeostasis of the CNS and regulation of metabolism and circadian rhythm would not be elucidated until many years later (Abbott et al. 2009). Recently, the study of the BBB has expanded to its role in toxicant-induced neurodegeneration (Zheng 2001). Given that the BBB is the frontline defense of the brain from peripheral insults, any disruption of its barrier function can lead to significant changes in CNS function leading to abnormal function of many physiological processes.

The conventional measure of BBB function is barrier "permeability" or how freely substances can pass through the BBB. Thus, BBB permeability has been used as a biomarker for many diseases of the brain and CNS. Different platforms and approaches have been used to study the BBB, each with its own advantages and shortcomings. In vivo, BBB permeability is measured by systemic injection of dyes and imaging, whereas in vitro, BBB permeability is assessed in artificial platforms that mimic the natural structure and function of the BBB. The difficulty with creating an in vitro BBB model is due to its complex interaction with cellular components of the CNS. However, the use of these artificial BBB platforms to measure BBB function as a biomarker in the field of neurotoxicity has grown rapidly due to the significant role of the BBB in regulation of brain function.

In this chapter, we introduce the structure and function of the BBB, discuss its role in toxicant-induced neural dysfunction, and elucidate the current in vivo, ex vivo, and in vitro methods used to assess the effects of toxicant exposure on BBB permeability.

Overview of the Blood-Brain Barrier

The BBB covers the entire neurovascular network except the pituitary gland, the median eminence, the area postrema, the preoptic recess, the paraphysis, the pineal gland, and the endothelium of the choroid plexus (Laterra et al. 1999). In these brain regions, lack of a BBB aligns with the need for direct sensing and releasing of circulating factors and quick communication with the periphery (Ferguson 2014). For the rest of the brain, the BBB interacts with the end feet of astrocytes, pericytes, neurons, and microglia to form what is known as the neurovascular unit (NVU) (see Fig. 1; Obermeier et al. 2013; Ahn and Kim 2021). As part of the NVU, the BBB

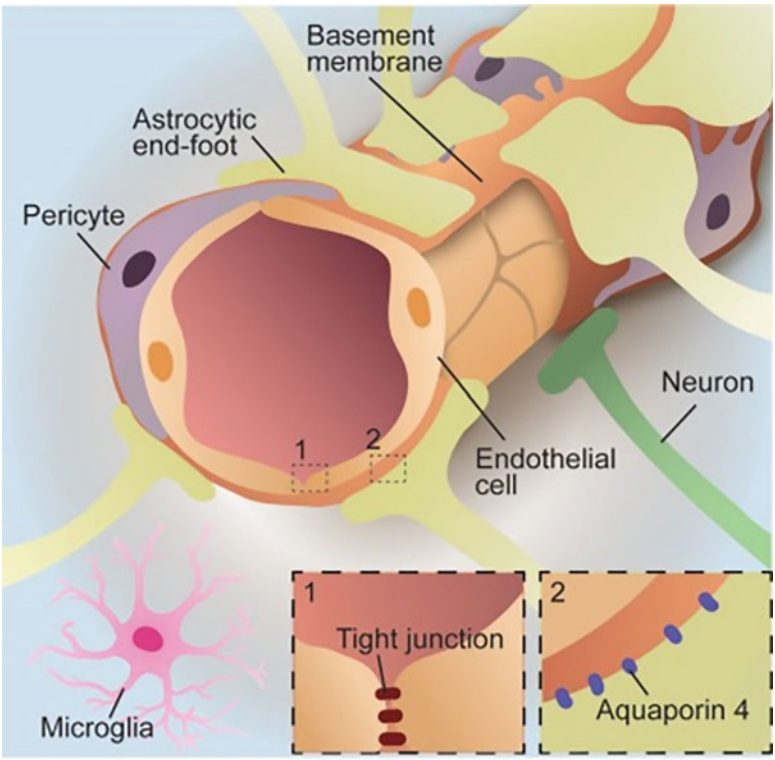

Fig. 1 The neurovascular unit. Depiction of the blood-brain barrier (BBB) in the context of the neurovascular unit (NVU). The synergistic interaction among the components of the NVU controls the paracellular and transcellular transport of molecules between the brain and the blood. (Reprinted from Ahn and Kim 2021 with permission)

prevents unwanted molecules from crossing from the blood into the brain while also recruiting and transporting the necessary nutrients from the blood into the brain. The synergistic interaction of the components of the NVU modulates the permeability of the BBB through regulation of tight junction (TJ) proteins and transport proteins expressed by the brain microvascular endothelial cells (BMECs) of the BBB (Obermeier et al. 2013). See Fig. 2 for a summary of paracellular and transcellular transport mechanisms of the BBB.

The BBB has hallmark functions of limited paracellular permeability due to the presence of tight junction complexes between adjacent BMECs that result in the absence of fenestrae (Mahringer et al. 2011; Ronaldson and Davis 2015). Tight junction proteins (occludin, claudins, cingulin, and membrane-associated guanylate kinase TJ proteins) and adherens junction proteins (cadherins, platelet endothelial cell adhesion molecule, and junctional adhesion molecules) expressed by BMECs bridge the gap between adjacent cells, making the BBB selectively impermeable to large polar molecules while allowing small lipophilic molecules to pass through the junction between the adjacent BMECs (Wong et al. 2013; Zhao et al. 2015).

In addition to TJs of the BBB, transport proteins expressed by BMECs play an important role in the maintenance of the milieu of the CNS via transcellular transport. Small, polar molecules that are essential for brain function, such as

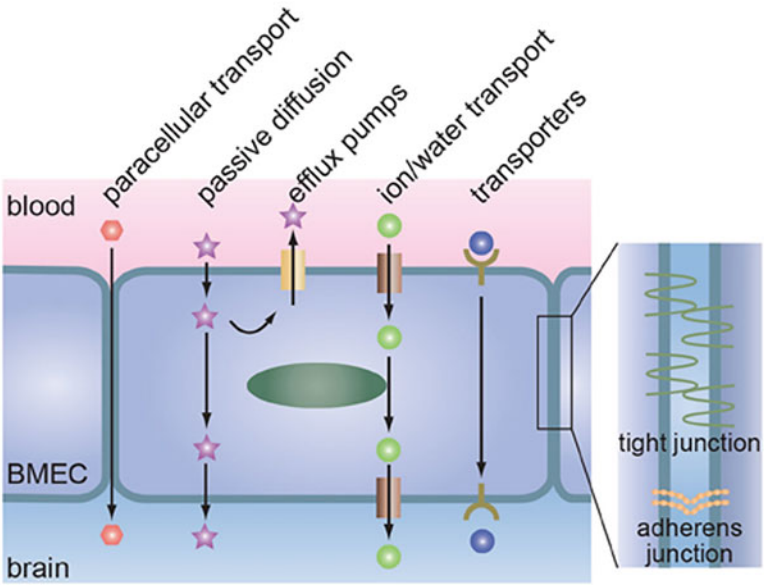

Fig. 2 Transport mechanisms of the blood-brain barrier. Paracellular and transcellular transport mechanisms of the blood-brain barrier (BBB). Paracellular transport occurs at the junction between the endothelial cells (ECs) of the BBB and is regulated by tight junction and adherens junctions. Transcellular transport occurs at the membrane of the ECs through passive diffusion, efflux pumps, ion/water channels, and other active transporters. (Reprinted from Wong et al. 2013 in accordance with Creative Commons Attribution)

glucose, amino acids, and nucleosides, cross the BBB via carrier-mediated transport mechanisms such as solute carrier transporters that are substrate-specific (Wong et al. 2013). Larger molecules, such as proteins, traverse the BBB through receptor-mediated transport mechanisms involving the binding of macromolecule ligands to the receptors on the endothelial surface (Kadry et al. 2020). Unwanted molecules, metabolites, and xenobiotics are transported from the brain into circulation via adenosine triphosphate (ATP)-binding cassette (ABC) transporters which are efflux pumps driven by ATP hydrolysis (Wong et al. 2013; Ronaldson and Davis 2015; Zhao et al. 2015; Kadry et al. 2020; Profaci et al. 2020). Members of the ABC transporter superfamily include P-glycoprotein (Pgp), breast cancer resistance protein (BCRP), and multidrug resistance-associated proteins (Mrps) (Wang et al. 2011a). Additionally, aquaporin 4 (AQP4) channels expressed in astrocytes play an important role in water permeability of the BBB (Bonomini et al. 2010).

TJ complexes and transport proteins of the BBB develop early in embryonic development in mammals (Hajal et al. 2021), with the BBB becoming fully functional in utero to protect the developing brain (Goasdoué et al. 2017). This reflects the importance of a properly functioning BBB early in development and into adulthood.

Blood-Brain Barrier Dysfunction as a Biomarker of Toxicity and Disease

When the BBB is damaged and becomes permeable to factors that are damaging to the CNS, neurodegeneration and severe neurological disease can ensue (Obermeier et al. 2013; Sweeney et al. 2018). A primary effect of increased BBB leakiness is the extravasation of immune cells/leukocytes into the brain causing neuroinflammation that may lead to brain lesions and white matter hyperintensities (WMHs) associated with memory loss (Gupta et al. 2018; Shalev et al. 2009; Abbott and Friedman 2012; Profaci et al. 2020). Additionally, impaired BBB function has been associated with stroke, brain tumors (Shalev et al. 2009), Alzheimer's disease (Montagne et al. 2017), multiple sclerosis, microglia tumors, amyotrophic lateral sclerosis, Parkinson's disease, and epilepsy (Zheng and Ghersi-Egea 2020). Oftentimes, it is unknown whether BBB dysfunction precedes neurodegeneration or vice versa; however, the breakdown of the BBB has been acknowledged as a gateway to the development of neurological and psychological disorders (Zheng and Ghersi-Egea 2020; Erickson et al. 2020).

While most environmental toxicants can bypass the BBB and negatively affect the brain tissue, there is strong evidence that toxicants directly act on the BBB and the NVU to induce neurotoxicity. The impact of toxicants, whether it be via neuroinflammation to induce BBB dysfunction, or direct insult upon the BBB, is undeniable, making BBB function a viable biomarker of toxicity. To follow is an in-depth discussion of toxicants that have been shown to induce neurotoxicity by directly impacting BBB permeability.

Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are chemicals that are naturally found in coal, crude oil, and gasoline but are also made when these substances and others such as tobacco, wood, food, and garbage are burned (CDC 2017). PAHs are characterized by the number of aromatic rings, ranging from two to ten rings. Due to their lipophilic structure, PAHs can easily cross the BBB and damage the brain causing behavioral changes and neurodevelopmental issues (Mortamais et al. 2017). Recently however, PAH-containing pollutants such as diesel fuel exhaust have also been found to disrupt BBB function directly (Hartz et al. 2008; Heidari Nejad et al. 2015; Oppenheim et al. 2013).

Benzo[a]pyrene (BaP), a PAH that is found in diesel fuel, crude oil, and partially combusted organic material, is able to cross the BBB without active transport to cause lipid and DNA damage in brain tissue. This damage can lead to learning and memory impairment and cause an increase in anxiety (Das et al. 2019). While BaP is able to easily cross the BBB, it has also been shown to directly alter the permeability of the BBB in vitro. A 2019 study demonstrated that, when BMECs representing the BBB in vitro were exposed to high levels of BaP, permeability of the BMEC monolayer was significantly increased, while exposure to lower levels of the BaP caused permeability to decrease (Ho and Burggren 2019). This indicates an important dichotomous concentration-dependent response of the BBB to toxicants such as BaP. While BBB leakiness is often associated with disease states as it can allow harmful substances to enter into the brain, a decrease in permeability may have unexplored negative repercussions in vivo (Zhang et al. 2016; Souza et al. 2016).

Dioxins

Dioxins are a class of toxicants that include polychlorinated dibenzodioxins, dibenzofurans, and biphenyls. The compounds belonging to these chemical families are structurally similar, composed of two benzene rings joined via one carbon bond, one oxygen bond, or two oxygen bonds. The main contributing factors to the production and environmental distribution of dioxins are industrial activities, waste incineration, and the burning of fuels (Kakeyama and Tohyama 2003). Dioxins have been reported to have long-term retention within the human body and have the potential to mediate variable toxic effects on different tissues (Kakeyama and Tohyama 2003).

Dioxins and other planar aromatic hydrocarbons are high-affinity ligands for the aryl hydrocarbon receptor (AhR), a DNA-binding transcription factor expressed in BMECs and astrocytes of the NVU. When bound with a ligand, AhR translocates to the nucleus, heterodimerizes with AhR nuclear translocator, and regulates expression of drug-metabolizing enzymes and xenobiotic transporters of the cell. In vivo and in vitro studies have reported that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), one of the most toxic dioxins, regulates expression of xenobiotic metabolizing enzymes and xenobiotic efflux pumps via an AhR-mediated mechanism in the BBB (Wang et al. 2011a; Miyazaki et al. 2016). Other in vitro studies suggested

that TCDD affects the expression of TJ proteins and, as a result, disrupts the formation and function of the BBB (Miyazaki et al. 2016). Along with data showing that exposure to TCDD is associated with cognitive impairment in animals and humans (Kakeyama and Tohyama 2003), it is likely that TCDD-induced BBB toxicity underlies the observed neurological deficits.

Perfluoroalkyl Substances

Perfluoroalkyl substances are another class of environmental toxicants that pose significant health concerns. Perfluoroalkyl substances are fully fluorinated carbon chains with varying functional groups. Perfluorooctanesulfonic acid (PFOS) is a perfluoroalkyl substance synthetically produced as a surfactant and repellant in a variety of commercial products, making it highly prevalent in the environment (Butenhoff et al. 2006; Wang et al. 2011b; Yu et al. 2020). PFOS has been reported to accumulate in serum and tissues of humans and wildlife (Butenhoff et al. 2006). PFOS enters the circulation, distributes in various tissues, and induces adverse effects on development and the neuroendocrine system (Wang et al. 2011b).

Yu et al. (2020) investigated the effects of PFOS exposure on the crosstalk between endothelial cells (ECs) and astrocytes in mice. In PFOS-treated mice, the presence of PFOS in the cerebral cortex was associated with astrocytic hypertrophy, upregulated markers of cerebral damage and astrocytic activation, and neuroinflammation. Gene expression of TJ proteins was decreased, and expression of proteins related to damage and stress was increased in a dose-dependent manner. Furthermore, in vitro examination of the effects of PFOS treatment on human glioblastoma U87 cells supported the in vivo experiments in that expression of TJ proteins was decreased and expression of damage-related proteins was increased (Yu et al. 2020). Supporting these findings, other studies that utilize in vitro BBB models demonstrated that PFOS contributed to decreased expression of TJ proteins and increased permeability of the BBB monolayer (Wang et al. 2011b).

Heavy Metals

Heavy metals such as arsenic, lead, and cadmium are abundant in the environment and can have dire consequences on human health. Exposure to heavy metals has been linked to an increased risk of developing Alzheimer's disease (AD) or the acceleration of the disease progression (Zheng et al. 2003). For example, low levels of arsenic, an industrial byproduct found in contaminated water, soil, and food, are associated with an increased incidence of AD (Rahman et al. 2021) as well as lower intelligence, memory impairment, and other neurological issues (Ramos-Chávez et al. 2015). Lead (Pb^{2+}) poisoning can have serious public health complications, as seen in the Flint Water Crisis (Ruckart et al. 2019). Studies have shown convincing evidence that exposures to lead early in life can lead to neurodegeneration with age (Sanders et al. 2009). Mounting evidence shows cadmium playing an etiological

role in the developments of AD as well as other neurodegenerative disorders (Jaishankar et al. 2014; Wang and Du 2013).

Although the consequences of heavy metal exposure on neurological function are well described, little is known of the effects of these toxicants on BBB permeability. Only a handful of studies address heavy metal toxicity on the BBB. Cadmium can bypass the BBB by entering the brain through olfactory neurons. Once in the brain, cadmium increases excitotoxicity and oxidative stress, increasing neuroinflammation which can damage the BBB (Wang and Du 2013). Arsenic has been shown to disrupt barrier function by altering the localization of TJ proteins of ECs and downregulating the expression of CD200, an inflammatory marker in neurons (Sherwood et al. 2013; Singh et al. 2019). Lead can directly cause oxidative stress resulting in apoptosis and excitotoxicity in the CNS by altering neurotransmitter functionality and other cells in the BBB increasing its permeability (Lanphear 2015; Jaishankar et al. 2014).

Airborne Toxicants and Pollutants

Exposure to airborne pollutants at a young age can lead to problems with brain development and neurodegeneration (Brockmeyer and D'Angiulli 2016). Prominent airborne pollutants measured in the air quality index include particulate matter, ozone, carbon monoxide, sulfur dioxide, and nitrogen oxide (Brockmeyer and D'Angiulli 2016). Chronic exposure to poor air quality can cause increased BBB permeability, neuroinflammation, WMHs, and cell loss (Brockmeyer and D'Angiulli 2016; Oppenheim et al. 2013). Exposure to high levels of vehicle emissions alone can have drastic impacts on the BBB and brain health. A 2013 study found that inhalation of vehicle emission increases the expression of a family of endopeptides which degrade TJ proteins in the BBB (Oppenheim et al. 2013). Exposure to air pollutants has also been shown to increase inflammatory markers in the brain leading to demyelination, neurotoxicity, and increased BBB damage (Brockmeyer and D'Angiulli 2016; Oppenheim et al. 2013).

Approaches to Studying BBB Permeability

Postmortem and In Situ Assessment

One of the earliest methods used to assess BBB permeability is the postmortem visualization and quantification of transport of dyes and labelled molecules into the brain after intravenous injection of these compounds. This approach has been used in BBB research since the discovery of the BBB by Ehrlich and has continued to be used today. Labelled markers or dyes are injected intravenously into the experimental animal, after which the animal is euthanized and the brain is excised for in situ visualization and/or quantification of the dye present in tissue. A variety of dyes and labelled molecules have been used to measure paracellular and transcellular

permeability of the BBB (Saunders et al. 2015). Commonly used molecules include Evans blue dye, horseradish peroxidase (HRP), sodium fluorescein, and dextran, each with their own unique properties.

Evans blue dye is often used for assessing transport across the BBB due to the ease by which it can be visualized in situ, as well as via colorimetric spectrophotometry (Saunders et al. 2015). HRP is another commonly used agent in early studies to elucidate the ultrastructure of the BBB and ascribe barrier properties to the TJ complexes in adjacent ECs (Saunders et al. 2015). Sodium fluorescein has a small molecular weight of 376 Daltons, significantly lower toxicity than Evans blue and HRP, and weak protein-binding capabilities (Saunders et al. 2015). Dextrans, branched polysaccharides composed of glucose, are labelled with either a fluorophore or biotin for ease of visualization and are available in a range of molecular sizes for use in quantitative studies of BBB permeability. Radiolabelled sucrose and inulin are also used to assess permeability of BBB (Raja et al. 2018).

Exogenous molecules that are known to utilize specific transport proteins for extravasation into the brain can be used for a more focused assessment of transcellular BBB function. For example, the use of verapamil, a known substrate for Pgp in the BBB, has been used to investigate the effect of TCDD-induced BBB dysfunction. In situ brain perfusion experiments were performed to measure the net transport of verapamil across the BBB by quantifying the accumulation of radiolabelled verapamil, in control and TCDD-exposed rats (Wang et al. 2011a). Alternatively, the use of tetramethylrhodamine biocytin, a molecule that can only cross the BBB when tight junctions are disrupted, has been used to specifically investigate paracellular permeability of the BBB (Knowland et al. 2014).

In human studies, endogenous blood-derived proteins such as fibrin, thrombin, albumin, immunoglobulin G, and hemosiderin are quantified in brain tissue postmortem as a sign of BBB dysfunction (Sweeney et al. 2019). Also, accumulation of circulatory cells in the brain including red blood cells, peripheral macrophages, and neutrophils has been used as indicators of disruption of BBB function (Sweeney et al. 2019). Concurrent measurement of BBB degeneration characterized by dysregulated transport and structural degradation of the NVU further supports the validity of using postmortem assessment of BBB dysfunction (Sweeney et al. 2019). The primary limitation of the postmortem approach in humans is that only the end stages of disease and dysfunction can be captured (Raja et al. 2018).

In Vivo Neuroimaging

With an increasing number of neurological diseases attributed to BBB dysfunction, in vivo examination of BBB permeability has become increasingly important (Raja et al. 2018; Saunders et al. 2014). Methods to assess and study BBB permeability with in vivo neuroimaging have improved with novel instrumentation and powerful computing capacities (Raja et al. 2018). Standard imaging techniques, such as computed tomography and magnetic resonance imaging (MRI), enable the detection of prominent and rapid disruptions in the BBB as observed in tumors or stroke. Less

prominent disruptions of the BBB, such as increased permeability caused by toxicant exposure, are only detectable by these current methods when measured for longer durations making them difficult to detect in clinical settings.

MRI techniques have wide applications and can be used to measure BBB leakiness. In a standard MRI, a strong magnetic field is created which forces protons in the target region to align. This magnetic field is pulsed, and the sensors in the MRI are able to detect the energy that is released when the magnetic field is off, and the protons are able to relax to their natural alignment. The tissue type determines the amount of time it takes for protons to realign and the amount of energy they release translating to the contrast image that appears after a scan (Hashemi et al. 2012).

The preferred MRI method for visualizing an increased BBB permeability utilizes paramagnetic contrast agents (Raja et al. 2018). Paramagnetic compounds normally do not cross the BBB. In cases of disrupted BBB integrity, the contrast agents are able to diffuse across into the brain. K_i, or the volume-transfer constant, quantifies the regional BBB permeability based on the resulting MRI image of the contrast agent (see Fig. 3). MRI has been applied to detect BBB breakdown in patients with mild cognitive impairment and early AD (van de Haar et al. 2016; Raja et al. 2018; Sweeney et al. 2019).

Positron-emission tomography (PET) is an imaging technique which requires the use of a radiotracer. A radiotracer is a radioactively tagged substance that is injected intravenously. During the scan, the tagged molecule can be tracked to measure perfusion of the molecule into different tissues. Radiotracers are designed using

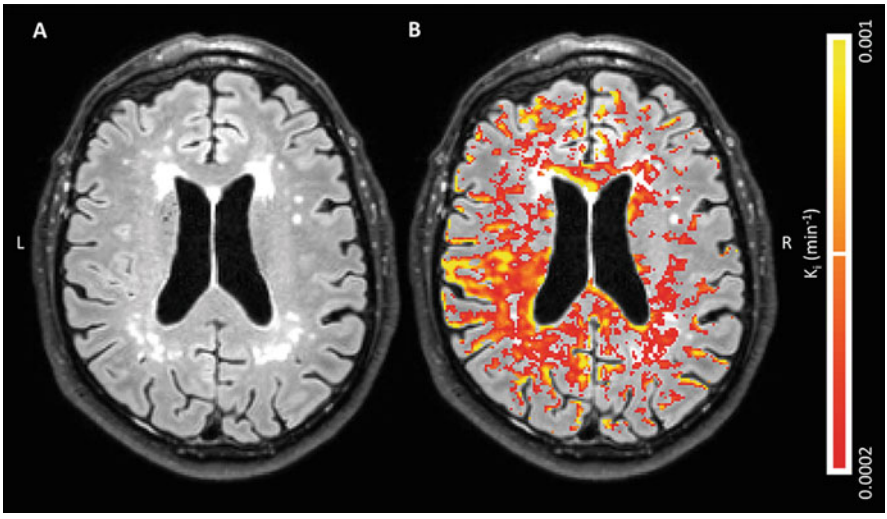

Fig. 3 Magnetic resonance imaging to detect blood-brain barrier leakage. Magnetic resonance imaging (MRI) of a patient that has been diagnosed with Alzheimer's disease. An image of the patient's brain (**a**) is overlaid with leakage rate values (**b**). The coverage and intensity of leakage are determined by color indicating leakage rate (K_i). (Reprinted from van de Haar et al. 2016 in accordance with Creative Commons Attribution)

molecules that would not be able to pass the BBB under normal conditions but are able to leak into the CNS with increased permeability. The amount of tracer that is present in the CNS can be used to quantify BBB permeability (Okada et al. 2015; Kessler et al. 1984). While the levels of radiation are extremely low, PET scans are considered more invasive than MRI scans and are therefore less preferable and used less frequently in human patients (Ollinger and Fessler 1997); therefore, this method may not be a viable option to assess BBB function as a biomarker of toxicant-induced neurotoxicity.

Ex Vivo Resected Blood Vessel Assay

The resected vessel assay is an ex vivo method with useful applications in BBB studies that examine the function of the membrane-bound efflux pumps (Wong et al. 2013). In this method, a resected brain capillary is placed in a dish with a buffer solution. A transporter-specific and fluorescently labelled solute of interest is then introduced to the media surrounding the capillary vessel, and the solute uptake is measured by recording the degree of fluorescence within the lumen of the vessel. As a result, the transport activity of select efflux pumps such as Pgp and BCRP can be determined (Wong et al. 2013).

Wang et al. (2011a) used the resected blood vessel platform to demonstrate that the toxicant, TCDD, alters normal transport function of the BBB. Transport assays were performed on isolated rat brain capillaries using a confocal microscopy-based method that measures the luminal accumulation of transporter-specific fluorescent markers as a function of the respective transporters. In one set of experiments, transport assays were performed using isolated rat brain capillaries exposed to different concentrations of TCDD. The results indicated that treatment with TCDD increased transport activity and expression of Pgp. In another set of experiments, laboratory rats were dosed with TCDD prior to euthanasia and blood vessel resection. Then, transport assays were performed using the isolated rat brain capillaries of control and TCDD-dosed rats. The results indicated that rats treated with TCDD displayed increased expression of Pgp, Mrp2, and BCRP which was in line with altered transport activity.

In Vitro Platforms

In vitro models of the BBB have been created to mimic physiological features of the BBB as it exists in the body. These platforms strive to provide robust avenues by which to readily investigate BBB permeability. Needless to say, the BBB is dynamic and complex; therefore, it can be challenging to replicate in vitro. A review of the literature reveals a wide variety of platforms that vary based on the following characteristics: (1) two-dimensional or three-dimensional, (2) monoculture or polyculture, (3) paracellular or transcellular permeability, and (4) static or dynamic.

The most realistic model would be a three-dimensional, dynamic, polyculture platform that is able to measure both paracellular and transcellular permeability. However, the more complex the model, the more challenging it is to create and use to obtain reliable and valid information on toxicity of toxicants.

The arrangement of the cellular components of the model may be two-dimensional as in a monolayer of BMECs grown on a plate or three-dimensional as in BMECs grown on scaffolding that provides a tubelike structure that simulates a blood vessel. Three-dimensional models provide the most realistic orientation of cells and are best for investigation of BBB function; however, three-dimensional models are relatively more technically challenging to create and use (Ferro et al. 2020).

Another important factor to consider is the type and assortment of cells used in each system. Primary hBMECs are the most realistic endothelial cells to use as they are what make up the endothelial layer of the BBB in humans. If human-derived cells are not obtainable, nonhuman animal brain microvascular endothelial cells can be used but are not phenotypically optimal for the assessment of toxicity in a human population. Human umbilical vein endothelial cells have also been used to model the BBB in several studies and exhibit many of the same properties as BMECs such as tight junction proteins and strong barrier function. Immortalized cell lines are also frequently used in BBB models due to their ease of use and low cost. However, due to the immortalization process, these cells lose many transport functions and may be a poor representation of the in vivo BBB. Although primary cells provide better genetic and physiological representations of the respective tissues of origin, immortalized cells are often easier to isolate with lower risk of contamination and have longer life spans. For a more in-depth review, see Ogunshola (2011), Vernon et al. (2011), Benson et al. (2013), Ferro et al. (2020), and Andjelkovic et al. (2020).

The permeability of the BBB can be recorded as (1) solute diffusion across the BBB or (2) transendothelial electrical resistance (TEER) of the BBB. Permeability to solutes is a measure of diffusion of molecules of a specific polarity and size as they pass through the modeled BBB. TEER, a measure of paracellular permeability, is the electrical resistance across the monolayer of cells that represent the BBB (Srinivasan et al. 2015; Hajal et al. 2021). Due to the TJs between BMECs, the BBB has relatively high TEER compared to endothelium of the rest of the body (Mahringer et al. 2011; Fu 2012; Sweeney et al. 2019; Profaci et al. 2020).

In static BBB models, cells are maintained in a stagnant medium. In dynamic BBB models, cells are exposed to a factor of shear stress that mimics the steady blood flow in living systems (Bagchi et al. 2019). Shear stress increases the expression of TJ proteins and reduces membrane permeability, suggesting that dynamic BBB models provide more realistic in vitro representations of the BBB (Bagchi et al. 2019). Nevertheless, there are various drawbacks of incorporating flow such as a longer time needed to reach a steady-state TEER and a high cell number requirement (Bagchi et al. 2019).

To follow, we discuss in depth the specific in vitro platforms used for measuring BBB function.

Electrical Cell-Substrate Impedance Sensing (ECIS)

ECIS technology is a popular two-dimensional platform for measuring barrier function continuously, in real time. In this system, ECs are grown in a monolayer in cell culture plates outfitted with electrodes. TEER is continuously measured over a period of time to track changes in paracellular permeability of the barrier (Maherally et al. 2018). This model is especially useful for observing changes in paracellular permeability in response to toxicant treatment, acutely or chronically. There are, however, several disadvantages to this model. Since this model generally uses a monolayer of endothelial cells to represent the BBB, it lacks the complex cell interactions of the NVU, which define the function of the native BBB. Without the presence of microglia, pericytes, and astrocytes, the barrier properties of the system are limited as the interaction with these other cell types has been shown to change the phenotype of endothelial cells.

ECIS technology has been used to capture the effects of a variety of toxicants such as crude oil, oil dispersant, and BaP (Ho and Burggren 2019). In these studies, primary mouse BMECs were cultured as a monolayer in a static environment. Continuous recording of TEER allowed for the elucidation of the time-course of BBB toxicity for the toxicants. Interestingly, each toxicant had a unique concentration- and time-dependent response to exposure, with lower concentrations generally causing an increase in TEER and higher toxicant concentrations causing a significant decrease in TEER.

Transwell Scaffold Model

One of the earliest and most common in vitro models of the BBB is the Transwell scaffold model. In this model, ECs are grown on a Transwell insert or a semipermeable membrane structure that separates the apical and basolateral side of the BBB (see Fig. 4; Wong et al. 2013). The porous membrane allows movement of small molecules through the monolayer of ECs. For BBB models, the input chamber of the

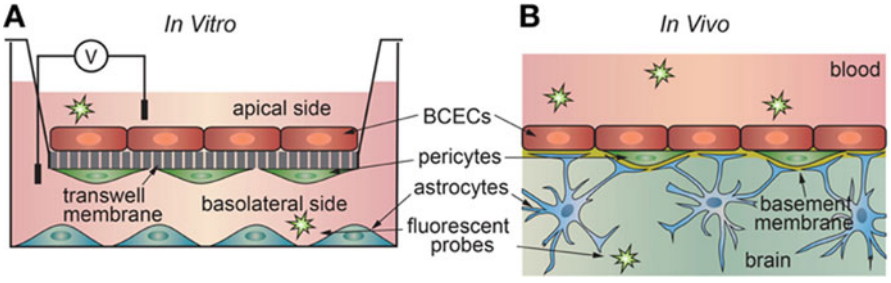

Fig. 4 In vitro Transwell scaffold model of the blood-brain barrier. Comparison of the Transwell scaffold model (**a**) to the blood-brain barrier as it exists in the body (**b**). The Transwell scaffold platform is a polyculture model that allows for the measurement of paracellular leakiness via transendothelial electrical resistance (TEER) and transcellular transport using fluorescent probes. *BCECs* brain capillary endothelial cells. (Reprinted from Wong et al. 2013 in accordance with Creative Commons Attribution)

insert represents the luminal side in contact with the blood, and the output chamber of the well represents the abluminal side in contact with the brain parenchyma.

Endothelial cells are grown either individually or with other NVU cell types. If more than one cell type is used, astrocytes or a co-culture of astrocytes and pericytes is grown on the bottom of the membrane. Once these cells are established, the membrane is flipped, and endothelial cells are grown on the other side of the membrane. The membrane allows for communication between the different cell types while still maintaining a similar structure to the BBB by allowing the endothelial cells to form an independent layer. The disadvantage of this model is that it is difficult to incorporate the factor sheer stress to make this a dynamic model (Gaston et al. 2017).

This model has been used to assess BBB permeability in response to toxicants. Miyazaki et al. (2016) investigated the role of TCDD exposure in disrupting the formation and function of the BBB using an in vitro rat BBB tri-culture Transwell model. This BBB model was based on co-culture of three different cell types of the NVU: primary cultures of rat brain capillary ECs, astrocytes, and pericytes. The cells were treated with TCDD at either 24 h post-seeding or 96 h post-seeding, representing the immature BBB and mature BBB respectively. TEER measurements were collected periodically, and the results indicated that TCDD only affected the permeability of the immature BBB. Expression of TJ proteins and glial cell line-derived neurotrophic factor (GDNF), a protein that promotes survival of astrocytes, was decreased with TCDD treatment.

Wang et al. (2011b) compared the effects of PFOS on BBB permeability and TJ structure of hBMECs. An in vitro monoculture model of the BBB, consisting of a monolayer of hBMECs, was treated with PFOS, and transport assays measuring HRP flux were performed. The findings indicated that PFOS increased permeability of the hBMEC monolayer, decreasing TEER and increasing HRP flux in a concentration- and time-dependent manner.

Organoids

Organoids, also known as spheroids, are small, three-dimensional spheres made of ECs, pericytes, astrocytes, and neurons that spontaneously form in culture. They are formed by co-culturing the cells under low adhesion conditions and allowing them to interact and form functional NVUs that possess many of the barrier functions of the native BBB. This model provides a relatively simple method of assessing the interaction between different types of cells in the BBB and the penetration of different compounds (see Fig. 5; Nzou et al. 2018; Bergmann et al. 2018). The disadvantages of this system are that even though there is interaction between the different cell types that make up the NVU, they are in a spherical conformation that lacks the structure and organization which may influence how the cells behave in vivo. There is also no possibility of incorporating sheer stress in this model which also limits the ability to accurately represent the in vivo BBB.

While BBB organoids are not frequently used in toxicant studies, they are useful in studying the permeability of CNS therapeutics. The ECs in BBB organoids have been shown to express TJs, molecular transporters, and drug efflux pumps. When a

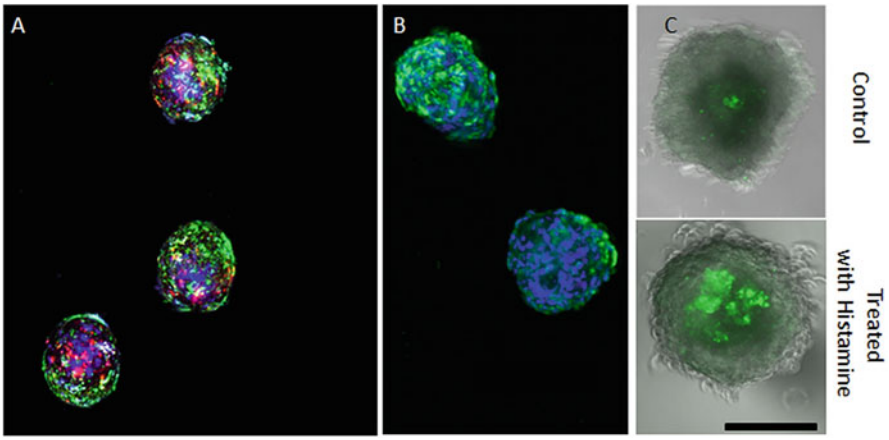

Fig. 5 In vitro spheroids representing the blood-brain barrier. Cellular and functional assessment of spheroids used to study neurotoxicity and neurological disease. Panel (**a**) shows spheroids composed of astrocytes and neurons (violet) encapsulated by endothelial cells and pericytes. Panel (**b**) shows staining of tight junction protein, ZO-1 (green). Panel (**c**) represents a functional experiment that demonstrates that spheroids that were treated with histamine, a chemical that disrupts endothelial barrier, allow significantly more proteins (green) into the spheroid than untreated spheroids. Scale bar, 300 μm. (Reprinted from Nzou et al. 2018 in accordance with Creative Commons Attribution)

drug that is known to cross the BBB is added to the media, it is able to permeate further into the organoid than drugs that are blocked by a healthy BBB. This can be visualized through microscopy or mass spectrometry (Bergmann et al. 2018).

Chips/Microfluidics

Chips and microfluidics devices are the newest frontier in BBB modeling and research (Deosarkar et al. 2015). These methods allow for a three-dimensional model that can incorporate shear stress via flow which will change the morphology of the cells and create more realistic conditions. Chips come in a variety of conformations, but in general they use separate microchannels to mimic the structure and organization of the BBB in the body. These channels maintain the morphology of the microvessels of the brain, and the ECs are organized so that they interact with NVU cells such as astrocytes and pericytes. The disadvantages of these models are that they can be labor intensive and expensive (Jiang et al. 2019). See Fig. 6 for illustration of different types of microfluidic chips (Fig. 6).

Since microfluidics devices and chips are relatively novel, they are not yet widely used in toxicant or BBB permeability studies. A recent publication (Li et al. 2020) developed a three-dimensional human BBB microfluidic chip model that examines the effects of indoor pollutants on oxidative stress-related biomarkers. In this model, fluorescently labelled human umbilical vein endothelial cells and astrocytes were treated with particulate matter alone or in conjunction with an antioxidant. Using fluorescence microscopy, the study revealed that cells treated with higher levels of

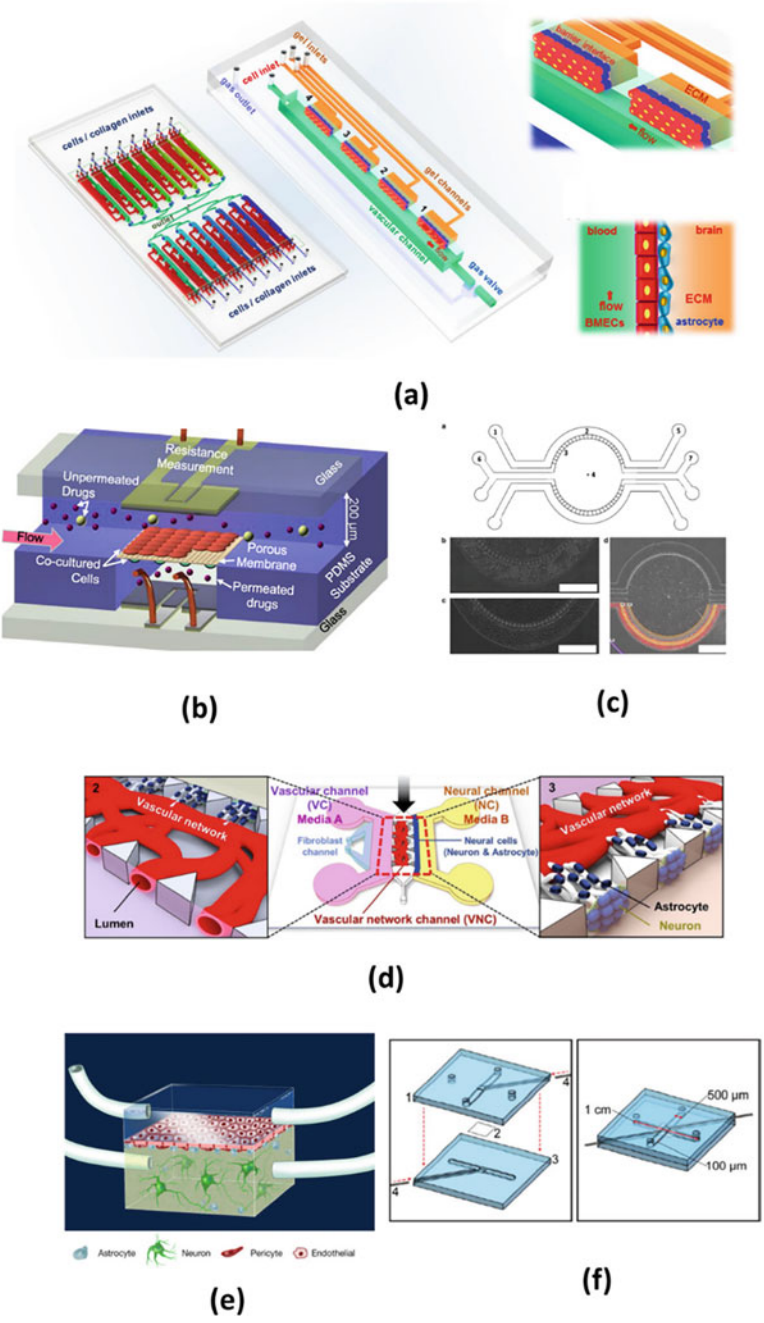

Fig. 6 Microfluidic chips. Examples of the types of microfluidic chips used to study the blood-brain barrier (BBB). (**a**) Three-dimensional chip with 16 different channels that each represents a functional neurovascular unit. (**b**) Chip that incorporates dynamic flow into a multilayered platform.

particulate matter had higher levels of astrocyte proliferation, a marker for neuroinflammation, while also increasing oxidative stress biomarkers in cells. While this study did not directly measure BBB permeability, oxidative stress and neuroinflammation are strong indicators of BBB dysfunction.

Other Platforms

There are other approaches that have used existing platforms in new ways to assess barrier function or even detect the presence of toxicants in the field. In a series of experiments, a team of scientists used ECIS technology and an enclosed biochip of the BBB as a detector for toxins in drinking water (Curtis et al. 2009b). The concept is that if the biochip measures a significant change in TEER after being exposed to a sample (e.g., water source), then the sample contains toxic pollutants. The first cell type used in these biochips was bovine pulmonary artery endothelial cells (BAECs) which were able to detect 7 out of 12 industrial chemicals found in polluted water samples. Later experiments showed that iguana heart (IgH-2) cells and bovine lung microvessel ECs were better able to detect toxicants, responding to 9 out of 12 as opposed to the 7 to which the BAECs responded (Curtis et al. 2009a, b, 2013). While the goal of these experiments was to determine the optimal cell types with the best cell viability in the field, these principals could be applied to detecting toxicants in the environment based on BBB permeability as a biomarker.

Conclusion

BBB function as a biomarker of toxicity is a budding field of investigation that promises vast insight into toxicant- and pollutant-induced neuropathology. Although further elucidation is needed of the mechanisms by which toxicants can impact BBB permeability, current experimental evidence demonstrates that BBB permeability is augmented after exposure to an array of environmental toxicants. More work is needed to solidify BBB function as a clinically relevant biomarker of neurotoxicity. Future endeavors should consider the use of neuroimaging techniques in humans to associate toxicant exposure to BBB permeability. These findings would greatly compliment the robust data already collected using the in vitro cell-based BBB models.

Fig. 6 (continued) (**c**) Gel-based chip composed of channels that allows for co-culturing of astrocytes and neurons in the center channel and endothelial cells in the outer channels. (**d**) Chip that recreates a vascular network and internal and external vascular microenvironments. (**e**) Two-chambered system where the endothelial cells are cultured on a porous polycarbonate membrane to allow for exchange between the chambers. (**f**) Two channels are separated by a polycarbonate membrane to represent the blood and brain compartments. (Reprinted from Jiang et al. 2019 in accordance with Creative Commons Attribution)

Applications to Prognosis and Other Diseases or Conditions

In this chapter, we have reviewed BBB dysfunction as a biomarker of toxicity and the impacts toxicants have on brain health. Dysfunction of the BBB, whether it be caused by external toxicants or internal factors, can have grave implications on human health and is therefore a useful biomarker for brain health as we age. The neurological damage resulting from a leaky blood-brain barrier can have lasting impacts whether it be a large breach such as a stroke or slow perfusion over time. Gradual memory loss as a result of leaks in the BBB may not be initially recognized; however, serious complications such as Alzheimer's disease, multiple sclerosis, microglial tumors, amyotrophic lateral sclerosis, and Parkinson's disease can also occur (Gupta et al. 2018). Ultimately, exposure to environmental toxicants can have lasting effects on human health. Symptoms may be immediate or appear many years later, making a reliable biomarker all the more necessary for early detection and prevention of future complications.

Mini-Dictionary of Terms

- **Blood-brain barrier**. A functional barrier of brain microvascular endothelial cells joined by tight junctions which forms around the microvessels of the central nervous system.
- **Central nervous system**. The brain and the spinal cord.
- **Dioxins**. Dioxins are highly toxic chemicals produced as byproducts of industrial activities, waste incineration, and fuel combustion that have a variety of toxic effects on human health.
- **Heavy metals**. A class of toxic metals and metalloids that have a high atomic weight such as mercury, cadmium, lead, aluminum, and arsenic.
- **Microfluidic devices**. Devices which contain and control small amounts of liquids in networks of microscopic channels.
- **Neurodegenerative disease**. A disease whose etiology is defined by neurodegeneration or the loss of function and eventual death of neurons.
- **Neurotoxicity**. Damage to the nervous system as a result of endogenous or external toxins or toxicants.
- **Neurovascular unit**. The neurovascular unit consists of the blood-brain barrier, astrocyte end feet, pericytes, neurons, myocytes, microglia, and perivascular macrophages and supports the blood-brain barrier allowing communication with the central nervous system.
- **Polycyclic aromatic hydrocarbons (PAHs)**. PAHs are a class of lipophilic chemicals found in fuel and partially combusted materials including benzo[a] pyrene, a carcinogenic chemical which can damage the blood-brain barrier.
- **Toxicant**. A pollutant or synthetically made substance that poisons or has deleterious effects on the health of living organisms.
- **Transendothelial electrical resistance**. A measure of the potential paracellular passage of ions by measuring the electrical resistance of a monolayer of cells.

- **Transport proteins**. Specialized proteins found in the blood-brain barrier which transport molecules in and out of the central nervous system. Different classes of transport proteins transport different types of molecules.

Key Facts of Blood-Brain Barrier Function as a Biomarker Platform in Toxicology: Impact of Toxicants

- Key facts of the blood-brain barrier
  - The neurovascular unit consists of astrocyte end feet, pericytes, neurons, myocytes, microglia, and perivascular macrophages and the blood-brain barrier.
  - The blood-brain barrier is formed from endothelial cells that are joined together by tight junction proteins and adherens proteins.
  - The discovery of the blood-brain barrier is credited to Paul Ehrlich who demonstrated that when dye is injected intravenously, every tissue in the body is stained except for the central nervous system.
  - Only small, positively charged, lipophilic molecules are able to cross the blood brain-barrier without transport.
  - External circulating factors such as toxins and internal factors such as neuroinflammation can damage the blood-brain barrier which is implicated in the development of neurodegenerative disorders.
- Key facts of in vitro models of the blood-brain barrier
  - The blood-brain barrier is difficult to mimic in vitro due to the complex interplay of factors contributing to its function in vivo.
  - The most common cell types used to study the blood-brain barrier in vitro are human and mouse brain microvascular endothelial cells, immortalized cell lines, and human umbilical vein endothelial cells.
  - Permeability of the blood-brain barrier is normally assessed in one of the two ways: transendothelial electrical resistance assays measure the paracellular resistance, and solute permeability assays measure diffusion across the barrier.
  - Models that rely on a monolayer of endothelial cells often lack the interaction with other cell types in the neurovascular unit which impact blood-brain barrier morphology.
  - Static models lack the shear stress caused by blood flow in vitro which impacts the morphology of the blood-brain barrier.
  - Cells grown in vitro require serum which is not present in the tightly controlled milieu of the central nervous system.
- Key facts of how environmental toxins impact the blood-brain barrier
  - Polycyclic aromatic hydrocarbons are lipophilic and can easily cross the blood-brain barrier and are found in partially combusted materials such as tobacco smoke, coal, and crude oil.
  - Dioxins are produced in industrial waste and combustion and can bind to the blood-brain barrier and alter transport functions.

- Perfluoroalkyl substances are used in a variety of substances and are useful surfactants and repellents that can break down the tight junction proteins in the blood-brain barrier leading to an increase in permeability.
- Heavy metals such as mercury, cadmium, lead, aluminum, and arsenic are prevalent in the environment and can cross the blood-brain barrier and impact its functionality while accumulating in the brain.
- Chronic exposure to poor air quality and airborne pollutants can cause increased blood-brain barrier permeability, neuroinflammation, and white matter hyperintensities which have all been implicated in neurological disorders and diseases.

Summary Points

- The blood-brain barrier protects and regulates the internal milieu of the central nervous system by selectively transporting nutrients, ions, and circulating factors from the blood.
- Increased permeability of the blood-brain barrier is implicated in the development of neurological and neurodegenerative disorders.
- Many environmental toxicants can directly damage the blood-brain barrier and cause increased permeability of the blood-brain barrier.
- There are different approaches used to assess the effects of environmental toxicants on the permeability of the blood-brain barrier, ranging from in vivo, ex vivo, and in vitro platforms.
- Blood-brain barrier function can serve as a reliable biomarker for toxicant-induced neuropathology.

Cross-References

► Biomarkers of Lead Exposure: Platforms and Analysis
► Lead and Aquatic Ecosystems, Biomarkers, and Implications for Humankind
► Linking Arsenic, DNA Methylation Biomarkers, and Transgenerational Neurotoxicity: Modeling in Zebrafish

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Anogenital Distance: Features, Measures, and Uses as a Biomarker for Toxicity In Utero

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Terje Svingen, Ulla Hass, Camilla L. Schwartz, and Sofie Christiansen

Contents

Abstract

The anogenital distance (AGD) refers to the distance between the anus and the external genitalia. In mammals such as humans, mice, rats, and cats, the AGD is close to twice as long in males than in females. This sexual dimorphism is caused by the hormone-dependent sexual development in utero, where high androgen levels in male fetuses promote masculinization of the reproductive system and the body more generally. The absence of high androgen levels in female fetuses helps promote feminization of the reproductive system. This dependence of the sex steroid hormone milieu on establishing the sexual phenotypes, including the length of the AGD, has established AGD as a noninvasive biomarker for androgen action during fetal development. Importantly, suboptimal androgen levels in

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utero can lead to various male reproductive disorders such undescended testes, hypospadias, and infertility later in life. Thus, a short male AGD signals not only suboptimal androgen action during development but is also associated with male reproductive disorders more broadly. This fact is exploited in animal toxicity studies to screen for chemical substances suspected to be harmful to human reproductive health but is also used in some human epidemiology studies.

Keywords

Reproduction · Perineum · Androgens · Testosterone · Anti-androgens · Endocrine disruption · Testis · Toxicity · Test guidelines · Anogenital distance · Sex development · Masculinization · Disorders · Risk assessment

Abbreviations

ADI	Accepted daily Intake
AGD	Anogenital distance
AGDi	Anogenital distance index
AR	Androgen receptor
DHT	Dihydrotestosterone
EDC	Endocrine-disrupting chemical
GD	Gestational day
LABC	Levator ani/bulbocavernosus
NOAEL	No observed adverse effect level
OECD	Organization for Economic Cooperation and Development
PND	Postnatal day
Sox9	SRY-box transcription factor 9
Sry	Sex-determining region of the Y chromosome
T	Testosterone
TDI	Tolerable daily intake
TG	Test guideline

Introduction

The anogenital distance (AGD) is sexually dimorphic in many mammals. In humans, rats, and mice, the AGD is about twice as long in males than in females. This difference between the sexes is a consequence of steroid hormone-dependent sexual development during fetal life. A high level of androgens during critical stages of fetal life leads to masculinization of the fetus, including the external genitalia, whereas the absence (or very low levels) of androgens leads to feminization of the fetus (MacLeod et al. 2010). The region between the anus and external genitalia, the perineum, is also sensitive to androgen levels and develop differently between the sexes (Schwartz et al. 2019). Because of this direct relationship between androgen action during development and AGD at birth, the AGD can be used as a biomarker for determining sex (as, for instance, with sexing newborn kittens and rats) but also

the level of masculinization in male offspring. If the male AGD is shorter than what would be expected based on mean control measurements (average in a population), it could indicate that the male offspring is not completely masculinized. This phenomenon can be taken advantage of in rodent toxicity studies aimed at determining potential endocrine-disrupting properties of chemical substances and drugs (Schwartz et al. 2019).

In this chapter, we provide an overview of how AGD is related to sexual development in humans and laboratory mice and rats. The main developmental processes are described, including current knowledge about the androgen mechanisms that are involved. We provide information about the use of AGD in rodent toxicity studies and its utility for chemical safety assessments, as well as comments about the use of AGD in human studies.

Sex Determination and the Early Stages of Sexual Development

In mammals, sex is initially determined by the pairing of sex chromosomes during fertilization: XY for males and XX for females. However, gonadal sex is not determined until much later, when XY genital ridges start to express the *Sry* (sex-determining region of the Y chromosome) gene. The expression of SRY prompts the upregulation of another important transcription factor, *Sox9*, which initiated the differentiation of the genital ridges into testes. In the absence of the Y chromosome, as in XX individuals, the *Sry* gene is not expressed in the genital ridges, and a separate genetic network is activated to initiate ovary differentiation (Svingen and Koopman 2013).

Shortly after the XY genital ridges have started to develop as testes, they organize into two distinct compartments: testis cords (future seminiferous tubules) which comprise Sertoli and germ cells and the interstitia which comprise steroidogenic Leydig cells alongside other cell types such as vasculature (Svingen and Koopman 2013). The Leydig cells rapidly start to synthesize androgens from precursor cholesterol, which will eventually be released into the circulatory system and transported throughout the body. Androgens, primarily testosterone (T) and dihydrotestosterone (DHT), act as ligands for the androgen receptor (AR), a nuclear transcription factor responsible for regulating transcription of numerous target genes (Chang et al. 1995; Davey and Grossmann 2016).

Androgen-Dependent Masculinization

As much as gonadal sex determination is under genetic control, the differentiation of the remainder of the reproductive system, and sexual phenotype more broadly, is heavily influenced by hormones. Although many molecular pathways are involved, the main pathway responsible for masculinization is androgen signalling. As mentioned earlier, the Leydig cells of the fetal testes produce androgens that are released into the circulatory system. The action of circulating androgens depends on the target

tissue, primarily whether cells express the AR or not (Davey and Grossmann 2016). T is converted into DHT by the enzyme 5α-reductase in several androgen-sensitive target tissues, which is important for masculinization effects as DHT is a more potent AR ligand than T. This local activation of AR in target sites then prompts masculinization of tissues and organs through the regulation of gene transcription (Fig. 1). This includes the presumptive muscle tissue of the perineum.

The presumptive perineal tissue of both male and female fetuses expresses AR in a subset of cells, but only in the presence of high levels of androgens will the tissue be instructed to differentiate into muscular cells and form the levator ani/bulbocavernosus (LABC) muscle complex. In other words, the LABC muscle complex normally only develops in the perineum of males. Equally important, androgen levels also determine the development of the external genitalia. Thus, the AGD is a function of androgen action on external genitalia and perineum during a critical window of fetal development (Schwartz et al. 2019).

In rats, the main period of androgen-dependent masculinization of specific tissues has been determined and is referred to as the masculinization programming window (MPW) (Welsh et al. 2008). This MPW is likely comparable across mammalian

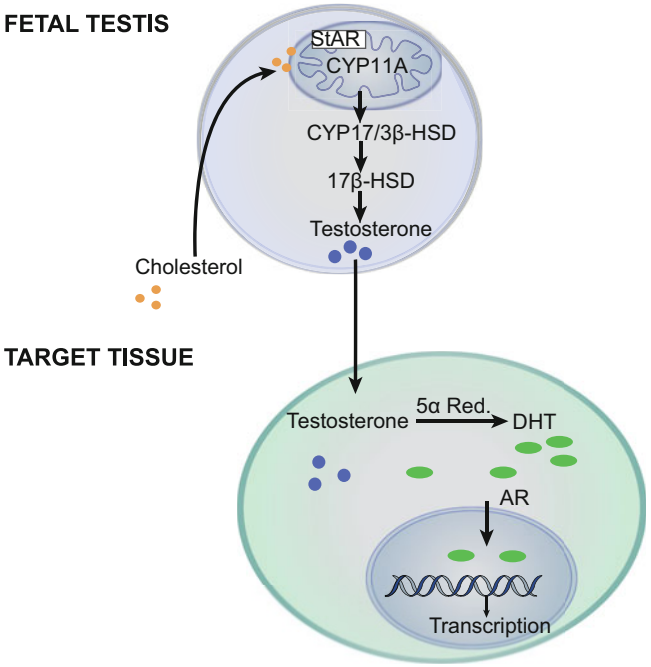

Fig. 1 Androgen signalling and male sexual development. In the developing fetus, androgens are synthesized by the testes, with testosterone subsequently released into circulation to reach target organs. Testosterone (T) is a ligand for the androgen receptor (AR), but in many androgen-sensitive tissues, T is converted into the more potent AR ligand dihydrotestosterone (DHT). Subsequently, DHT (or T) can bind to and activate the AR that translocate to the nucleus and regulate gene transcription

species, although the timing during gestation can differ. For instance, the MPW occurs towards the latter stages of gestation in rats and mice, but takes place around weeks 8–14 of gestation in humans (Sharpe 2020). Nevertheless, the important point here is that androgen action drives masculinization during fetal life and that this general masculinization effect can be retrospectively assessed by measuring the AGD.

Why Measure AGD

As already mentioned, the AGD is a broad biomarker used in rodent reproductive toxicity studies and some human epidemiological studies (Schwartz et al. 2019; Thankamony et al. 2016). Changes to AGD in such studies are of interest for two main reasons. Firstly, a change to AGD in rodent offspring following in utero exposure can say something about potential endocrine-disrupting properties of chemical substances. Secondly, altered AGD has been associated with various reproductive disorders. These points are summarized in Fig. 2 and discussed in more detail below.

AGD in Rodent Toxicity Studies

The most common application for AGD measurements is in male offspring where a shorter AGD indicates incomplete masculinization during development. Since (as outlined above) AGD is sensitive to androgen signalling, a shorter male AGD suggests compromised AGD action, i.e., a short male AGD is typically considered a measure of an anti-androgenic effect. There are many examples from rat toxicity studies where fetal exposure to chemical substances leads to a shorter male AGD, such as phthalates, pesticides including vinclozolin and procymidone, and several azole fungicides, as well as anti-androgenic including finasteride and flutamide (Schwartz et al. 2019). These various chemical substances have different mechanisms of action but have in common that they inhibit androgen signalling during the MPW. Mechanisms of action involve reducing T biosynthesis, inhibiting the conversion of T to DHT or by blocking AR activation. Hence, diverse chemical substances with diverse mechanisms of action can cause the same in vivo adverse effect – a short male AGD – which is why AGD is a tractable biomarker for anti-androgenicity in rodent toxicity studies.

A longer female AGD would typically indicate that the female offspring had experienced excess androgen action during fetal life. In other words, a female pup with a long AGD would be considered androgenized, and a chemical substance that would elicit such an effect would be categorized as androgenic. This can result in masculinized behavior but also phenotypes such as masculinized genitals, higher testosterone levels, disrupted ovulation, and polycystic ovary syndrome in woman (Abbott et al. 2005; Mira-Escolano et al. 2014). The challenge arises when the AGD in rodent studies turns out to be either longer in male offspring or shorter in female

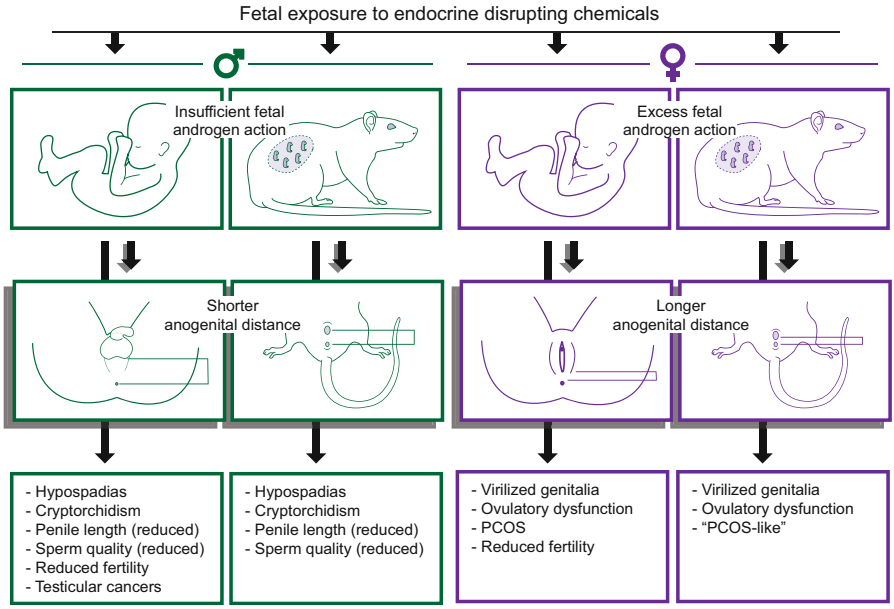

Fig. 2 Anogenital distance (AGD) is sensitive to early life exposure to endocrine disruptors and is a useful biomarker for reproductive disorders in rodents and humans. In males, androgen insufficiency leads to undervirilization of the fetus and various disorders of androgen-sensitive tissues. This relationship is clear from animal models, whereas human studies have established associations. In females, excess androgen signalling leads to virilization of the fetus and various disorders later in life. As in males, this relationship has been shown in animal models, whereas human studies have established associations

offspring. This is uncommon, but there are examples. As of yet, the underlying mechanisms for these atypical AGD responses are unclear, but it is important to note that they do occur and that they are not simply data anomalies in a single study.

AGD in Human Epidemiological Studies

In humans, AGD is used as a broad biomarker in epidemiological studies to establish associations between chemical substances and effects on reproductive development (Thankamony et al. 2016). In particular, a short AGD in newborn boys has been associated with in utero exposure to EDCs, most prominently phthalates (Bornehag et al. 2015). Notably, there are also many epidemiological studies that have not found association between a short male AGD and EDC exposures, which highlights the difficulty in establishing clear cause-effect relationships in humans. As humans are constantly exposed to a variety of chemical substances, it is very difficult to study the effect of single compounds on AGD outcomes. In animal toxicity studies, both

high and low doses are used to establish dose-response relationships, and the doses that elicit clear significant changes to AGD in the animals are often higher than what humans normally are exposed to. It is important to keep in mind, however, that factors such as interspecies toxicokinetic differences make it impossible to directly compare doses between humans and, for example, rodents. Regardless, the large number of chemicals that humans are exposed to, and the fact that chemical substances sharing molecular modes of action, can cause additive or synergistic effects. Changes to AGD in human subjects, small or not, are of concern.

Importantly, as mentioned above, the fact that a short AGD is associated with other reproductive disorders such as hypospadias, undescended testes, and poor sperm quality is what makes AGD an important biomarker in human epidemiological studies (Schwartz et al. 2019; Thankamony et al. 2016). For example, in boys a shorter AGD is associated with hypospadias and undescended testis (Hsieh et al. 2008, 2012; Jain and Singal 2013; Thankamony et al. 2014), whereas in men it has been associated with decreased fertility (Eisenberg et al. 2011), impaired semen quality (Mendiola et al. 2011), lower serum testosterone levels (Eisenberg et al. 2012), and testicular germ cell tumors (Moreno-Mendoza et al. 2020). These associations support the view that AGD is a valid biomarker in humans for male reproductive disorders often grouped under the testicular dysgenesis syndrome hypothesis, as suggested a decade and a half ago (Sharpe 2005).

How AGD Is Measured

Simply put, the AGD is obtained by measuring the distance between the anus and external genitalia (Fig. 3). In practical terms, however, there are some challenges in obtaining robust and reproducible data. There are also differences between how AGD is measured in rodents and humans, as described in the following sections.

AGD Measurements in Rodents

In rodent studies, the AGD is normally measured around the time of birth, between GD21 to PND4 as described in the OECD test guidelines (TG 414, 443, 421/422). These guideline measurements should adhere to the principles set out in the OECD guidance documents (GD 43 and GD 151), where it is stipulated that "a statistical significant change in AGD that cannot be explained by the size of the animal indicates effects of the exposure and should be considered in setting NOAEL (No Observed Adverse Effect Level)." In other words, a statistically significant change in AGD in rat offspring can be used to determine the NOAEL and thereby be used as the point of departure for setting safe exposure levels for humans (such as ADI and TDI).

Newborn male rats do not have a discernible scrotum. The external genitalia are still not fully developed at birth in laboratory rodents (rats and mice), with only a

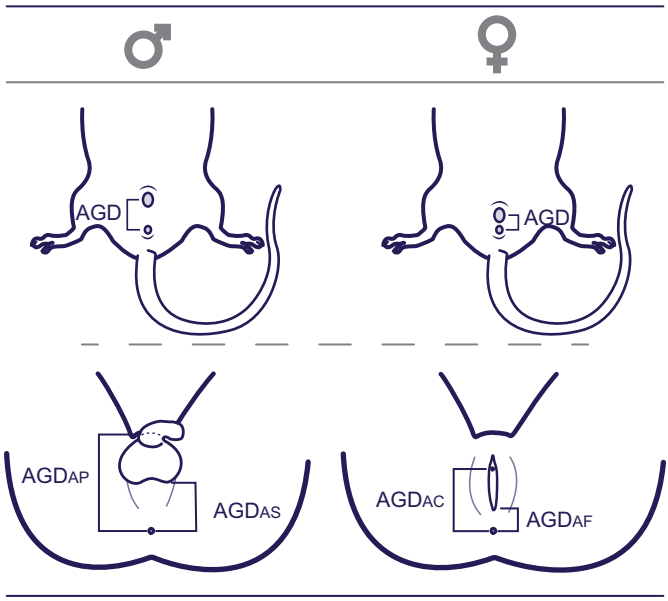

Fig. 3 Measuring anogenital distance (AGD) in rats, mice, and humans. In rats and mice, the AGD is measured as the distance between the anus and the insertion of the genital tubercle. In humans, there are different approaches for measuring the AGD, with two different measurements in males and females. In males, one measures the distance between the anus and the base of the scrotum (AGD_{AS}) or between the anus and the anterior insertion of the penis (AGDAP). In females, one measures the distance between the anus and the fourchette (AGD_{AF}) or between the anus and the anterior insertion of the clitoris (AGD_{AC})

genital tubercle apparent in both sexes in place of a penis or clitoris. In rats (and mice), the AGD is therefore measured from the base of the genital tubercle to the proximal end of the anal opening, typically using a dissecting microscope with a micrometer eyepiece or another sensitive method (Fig. 3). The birth/fetal weight should be measured at the same time, since there is a significant correlation between AGD and body weight (Gallavan et al. 1999). When analyzing AGD, the body weight should thus be accounted for in this assessment. For this purpose, the AGD index (AGDi) is calculated by dividing the AGD by the cube root of body weight. Body weight should also be included as a covariate in the statistical analysis of AGD (Gallavan et al. 1999; OECD 2008, 2018).

When measuring the AGD, the examiner should be blinded to study groups. That is; since AGD can be influenced by the way in which the examiner holds and stretches the animal under the microscope, it is imperative that the examiner does not become biased during measurements. This also stresses the point that AGD measurements should ideally be performed by the same person(s) for the entire study. Likewise, it is important that the examiner holds the pup as similar as possible

each time a measurement is recorded. For this, the pup should be kept face up in a horizontal position parallel to the eyepiece with the measuring scale. The examiner must be careful not to stretch the bodies, as this will increase the length of the AGD and lead to a variability in the results.

AGD Measurement in Humans

In 2005, an epidemiological study reported on possible association between fetal exposure to phthalates and short male AGD in humans (Swan et al. 2005). Since then, AGD has been reported in many epidemiological papers, especially studies with a focus on potential reproductive effects following intrauterine exposure to EDCs (Thankamony et al. 2016). As is the case in laboratory rats and mice, the AGD in newborn humans is also close to twice as long in males than in females (Salazar-Martinez et al. 2004).

A major challenge in using human AGD data collection has been a lack of good reference values or established baseline values. A significant step towards overcoming this challenge has been the work of the International AGD Consortium (IAC), which recently presented age-, sex-, and method-related reference levels for AGD based on measurements of more than 3,500 healthy infants (Fischer et al. 2020). According to the IAC, there are two main methods for measuring AGD in humans (Fig. 3). The shorter of the two measurements is from the anus to the junction between the perineum and the scrotum (AGD_{AS}) in boys and from the anus to the fourchette (AGD_{AF}) in girls, respectively. The longer of the two measurements is from the anus to the anterior insertion of the penis (AGD_{AP}) in boys and from the anus to the clitoris (AGD_{AC}) in girls. As in rodent studies, adjustments for body weight of the infant can be done, for example, by calculating the AGD/body mass index (BMI; kg/m^2) ratio (Fischer et al. 2020).

Final Remarks

The AGD has been used as a noninvasive biomarker for decades and will continue to be used in the future. For what purposes it will be used, however, may change. Currently, it is regarded a robust biomarker for reproductive toxicity studies in that a short male AGD in newborns indicates insufficient androgen signalling during critical stages of development, whereas a long female AGD indicates excess androgen signalling. With a clear association with other reproductive disorders in both sexes, in rodents and in humans, AGD has a significant value in evaluations of chemical substances for potential endocrine-disrupting activities. Beyond these facts, there are certain aspects regarding AGD measurements and interpretation that need careful consideration to avoid erroneous data recording and drawing of conclusion. We end by emphasizing three points related to this:

(i) Litter effects: In rodents as in humans, siblings are genetically more similar than the general population. Furthermore, in toxicity studies, the exposure scenario will be very similar within the one litter, whereas there can be greater differences between litters because of differences in dam ADME and so forth. Therefore, when performing statistical analyses on AGD data within large toxicity studies, the litter should be regarded as the statistical unit.
(ii) The actual measuring of AGD is challenging as it usually involves handling live animals under a microscope. The "stretch" of the animal upon measuring will influence the results; thus, it is imperative that each animal is held as similarly as possible each time a measurement is taken. If this is not done, the variation in the data could end up too large to observe "subtle" effects on changes to AGD. It is therefore strongly recommended to have the same person (s) perform all of the measurements within a complete study. Also, the person that measures AGD should ideally be blinded to exposure groups, as the measurement is susceptible to bias.
(iii) There is no current robust guidance on how to measure AGD in rodent toxicity studies. This is needed and should be developed and adopted across laboratories performing reproductive toxicity studies that include AGD data.

Application to Other Diseases or Conditions

In this chapter, we review the usability of anogenital distance (AGD) as a retrospective biomarker for disrupted androgen signalling during development. In males, a shorter AGD suggests insufficient androgen action, which has led to undervirilization effects. This extends to multiple organs and tissues, and there is a strong correlation between a short AGD and male reproductive disorders in rodents and in humans (e.g., hypospadias, cryptorchidism, infertility). In females, a longer AGD suggests excess androgen action during development, which has led to virilization effects. There is a strong correlation between a long AGD and female reproductive disorders (e.g., virilized genitals, PCOS, infertility).

Mini-dictionary of Terms

- **Anogenital distance**. The distance between the anus and external genitalia. This is a sexually dimorphic feature in many mammals, where the distance is longer in males than in females.
- **Androgens**. A group of steroid hormones that regulate development and maintain adult characteristics and functions across in vertebrates by acting through the androgen receptor. The major two androgens are testosterone and dihydrotestosterone.
- **Endocrine-disrupting chemical**. A chemical substance that can interfere with the endocrine signalling system and cause adverse effects. Most commonly through the EATS modalities (estrogen, androgen, thyroid, and steroidogenic).

- **Anti-androgen**. A substance that can inhibit androgen action, either by reducing androgen biosynthesis or blocking androgen receptor activation or signalling.
- **Hypospadias**. A birth defect in boys, and male rodents, where the urethral opening does not end at the tip of the penis, but instead somewhere on the underside of the head or shaft of the phallus.

Key Facts of Androgen-Dependent Sexual Development

In most mammals, the development of secondary sexual characteristics is dictated by androgen levels.

The testis is the main site for testosterone synthesis, so only male fetuses produce high levels during development.

The adrenal glands produce low levels of androgens, so females also produce lower levels of androgens.

High androgen levels during fetal life determine male sexual development.

Low androgen levels during fetal life determine female sexual development.

Summary Points

- In mammals, male sexual development initiates during fetal life and is critically dependent on androgens such as testosterone and dihydrotestosterone.
- The absence of high androgen levels in female fetuses allows for female sexual development to progress normally.
- The anogenital distance in mammals such as humans, rats, and mice is about twice as long in males than in females and is a biomarker of hormone-dependent sexual development in utero.
- Exposure to chemicals with anti-androgenic properties during fetal development prevents normal masculinization of the reproductive system and other tissues.
- A short anogenital distance in newborn males indicates incomplete masculinization and is linked to many reproductive diseases.
- A long anogenital distance in newborn females indicates virilization and is linked to various diseases.

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Micronucleus Assay in Lymphocytes for Human Biomonitoring and Clinical Studies

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Contents

Abstract

Micronuclei (MN) are originated when a whole or broken chromosome/chromatin lag at the anaphase of dividing cells and becomes separated from the main chromatin during telophase. The test to detect this structure in lymphocytes, the

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cytokinesis-block micronucleus cytome (CBMN-Cyt) assay, has been used for over 40 years and it is still being applied in new and promising areas of biology, health, and nanotechnology. It can be used to assess the genotoxic effect derived from occupational and environmental exposure or because of having a chronic disease. In addition, the test can be considered an indirect measure of prognosis in clinical studies. The present chapter reviews different studies that describe the formation of MN in lymphocytes and the associations of the increase of MN in individuals exposed environmentally and occupationally to different genotoxic agents. In addition, this chapter brings some results of this biomarker of genomic instability and its relation with different diseases.

Keywords

Micronucleus test · Genotoxicity · Lymphocytes · Binucleated cells · Occupational exposure · Environmental exposure · Chronic disease · Biomonitoring · Biomarkers · Genetic instability

Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
AT	Ataxia telangiectasia
AUD	Autoimmune diseases
BFB	Breakage-fusion-bridge
BN	Binucleate cells
CAD	Coronary artery disease
CBMN-Cyt	Cytokinesis-block micronucleus cytome
CKD	Chronic kidney disease
COPD	Chronic obstructive pulmonary disease
CRC	Colorectal cancer
CS	Cockayne's syndrome

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DNA	Deoxyribonucleic acid
DS	Down syndrome
FAL	Formaldehyde
GTS	Green tobacco sickness
HNC	Head and neck cancers
IARC	International Agency for Research on Cancer
MCI	Mild cognitive impairment
MN	Micronuclei
NBUD	Nuclear buds
NPB	Nucleoplasmic bridges
NSCL/P	Non-syndromic orofacial clefts and/or palate
NTD	Neural tube defects
PAH	Polycyclic aromatic hydrocarbons
PD	Parkinson's diseases
ROS	Reactive oxygen species
SIL	Squamous intraepithelial lesions
T2D	Type 2 diabetes
WS	Werner's syndrome
XP	Xeroderma pigmentosum

Introduction

Among the classical cytogenetic techniques used in human monitoring, the study of chromosomes through observation and counting of chromosomal aberrations in cells in metaphase stands out. This approach allows for a detailed analysis, but the complexity, the extensive handwork, and the presence of artifacts, such as the loss of chromosomes during the preparation of the slides, stimulated the development of a simpler methodology aimed at measuring chromosomal damage, such as the micronuclei (MN) test. MN are expressed in dividing cells that contain chromosome breaks without centromeres (acentric fragments) and/or entire chromosomes that are unable to migrate to the poles during mitosis and may lag in anaphase. Acentric chromatid and/or chromosomal fragments usually originate after extensive DNA damage such as double-strand breaks that, if misrepaired, result in asymmetrical chromosome rearrangements and exchanges (clastogenesis). Malfunction of the chromosome centromeres or kinetochore or damage to the mitotic machinery, which includes the mitotic spindle, necessary for chromosome segregation after DNA replication, can lead to the loss of entire chromosomes or chromatids (aneugenesis). In telophase, a nuclear envelope forms around chromosomes or fragments, which then unfold and gradually assume the morphology of an interphase nucleus, with the particularity of being smaller than the main nuclei of the cell (hence the term micronucleus), providing a reliable index of chromosome breakage and loss (Fig. 1) (Fenech 2007).

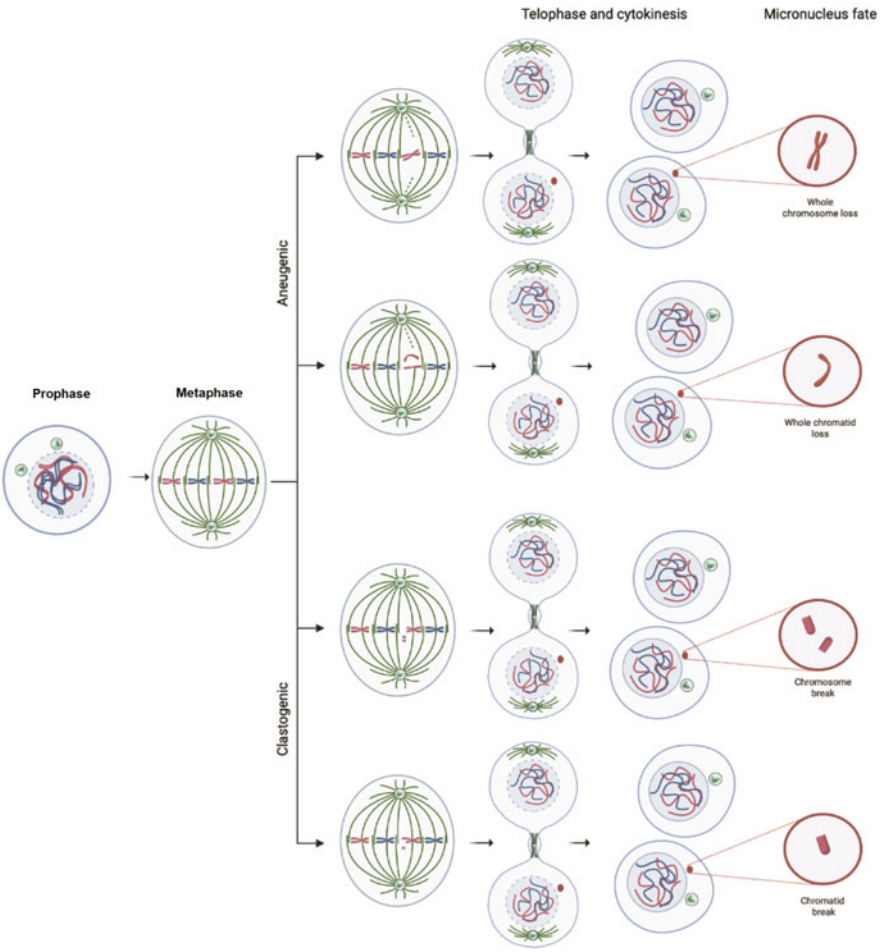

Fig. 1 Graphical representation of main mechanisms of micronucleus formation. MN can arise from whole lagging chromosomes and/or chromatids due to defects in centromeric DNA, in kinetochore proteins or assembly. Such events give origin to MN containing whole chromosomes and/or whole chromatids, classified as aneugenic defects. In the event of a clastogenic defect, MN originated from acentric chromosomes and/or chromatids, resulting in fragments due to DNA breakage episodes. Created with BioRender

What we know as the micronucleus test in humans began to be studied in 1891 (Fig. 2), by W.H. Howell, who described some characteristics in blood cells, such as spherical granules seen in erythrocytes and believed to be of nuclear origin associated with chromosomal damage. Later, J. Jolly's studies in 1901, and his refinement in some of Howell's conclusions, inferred that MN were remnants of red blood cell nuclei circulating in organs with pathological characteristics (karyorrhectic cells), which justified the inclusion of his name in the initial definition of MN, the Howell-Jolly corpuscles. A new description for MN occurred in the mid-twentieth

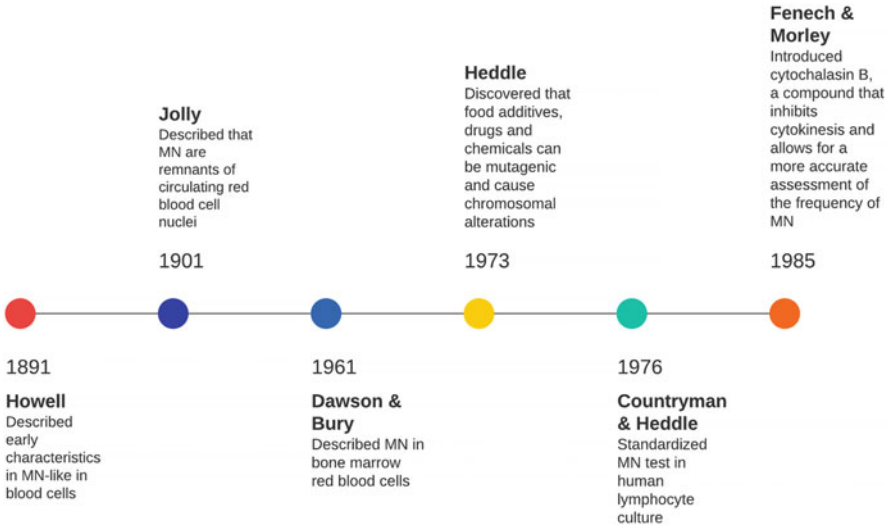

Fig. 2 An abbreviated timeline from the main moments in the development of the MN assay until reaching the lymphocyte test

century, when D. Dawson and H.P. Bury, in 1961, found MN in bone marrow red blood cells at different stages of pathologies. The idea that food additives, drugs, and chemicals can be mutagenic and cause chromosomal alterations in exposed people was already raised by J.A. Heddle in 1973. In 1976, P.I. Countryman and J.A. Heddle developed the MN test in human lymphocyte cultures. The researchers quickly found applications for in vitro testing as well as in vivo exposure studies for humans exposed to hazardous agents. The Countryman and Heddle's version of the MN test in human lymphocytes underwent some modifications proposed by M. Fenech and A. Morley around 1983 (Hayashi 2016). In the following years, M. Fenech and A. Morley found that the test did not discriminate between cells that had not divided yet and cells that had already divided. As MN can only be expressed after nuclear division, and as the proportion of dividing cells can vary between cell cultures, the standard MN test may be inaccurate. After testing several methods to solve the problem, the researchers realized that blocking cell cytokinesis, with the use of cytochalasin B, was the most suitable approach. In this method, dividing cells are inhibited from performing cytokinesis and, therefore, are easily recognized by their binucleate appearance. The method was considered to be simple and accurate, which is why it is still currently in use. The cytokinesis-block micronucleus cytome (CBMN-Cyt) assay protocol was published in detail in 2007 by Fenech (2007).

In addition to the mechanisms of MN formation, the MN test allows the assessment of the formation of nuclear anomalies such as nucleoplasmic bridges (NPB) and nuclear buds (NBUD) (Fig. 3). NPB are formed from dicentric chromosomes, and they can arise due to incorrect repair of DNA breaks, final telomere fusions, and defective separation of sister chromatids at anaphase following decatenation failure. The amplified DNA elimination process, DNA repair complexes, and possible

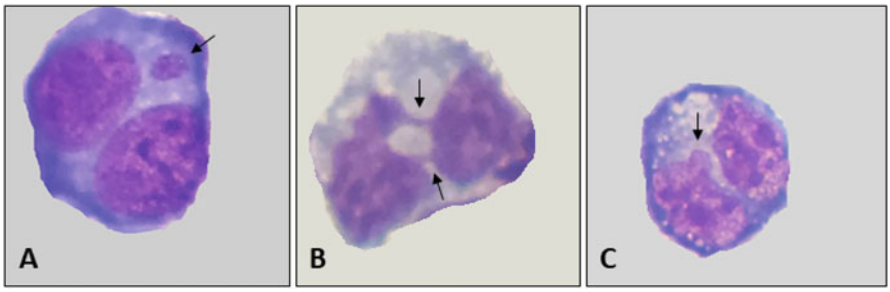

Fig. 3 Photomicrographs of the lymphocyte cells scored in the CBMN-Cyt assay. (A) binucleated (BN) cell with micronuclei (MN); (B) BN with two nucleoplasmic bridges (NPB); and (C) BN cells containing nuclear buds (NBUD). The cells were visualized by optical microscopy at 1000× resolution

excess chromosomes from aneuploid cells give rise to NBUD. In addition to NPB and NBUD, cell death and cytostasis are also biomarkers of DNA damage that can be observed using the MN test (Fenech 2007).

The genetic damage detected by the MN test can be caused by different environmental factors, occupational exposure, micronutrient deficiency, lifestyle, and genetic factors, as well as natural processes such as aging. The increased frequency of MN in humans has been associated with several types of pathologies, such as cancer, neurodegenerative and metabolic diseases, fertility, and birth and development defects (Bonassi and Fenech 2019; Franzke et al. 2020; Bolognesi et al. 2021; Dhillon et al. 2021; Knudsen and Kirsch-Volders 2021; Wagner et al. 2021).

The aim of the present chapter was to survey recent human biomonitoring and clinical studies that addressed the use of MN in lymphocytes in the assessment of exposure of humans to genotoxic and mutagenic agents as well as to related diseases.

Occupational and Environmental Monitoring

Occupational and environmental biomonitoring assess human exposure to chemicals, by measuring substances or their metabolites in bioindicators such as urine, hair, blood, and other tissues. The information generated through the data obtained from biomonitoring, associated with epidemiological, toxicological, and pharmacokinetic studies, help to determine the magnitude of exposure, susceptibility, and effects of these agents in the potential health risk (Council 2006).

Publication of occupational biomonitoring studies in the determination of chemical substances in bioindicators started in the 1890s, using techniques to measure the amount of lead or benzene metabolites in the blood and in urine (Angerer et al. 2007). New techniques to measure the concentration of chemical agents in human body fluids were introduced over time and occupational medicine was able to quickly detect very low concentrations of substances in the body.

Biomarkers are classified into three classes: exposure, effect, and susceptibility. Exposure biomarkers are used to measure the exposure to xenobiotic agents or their metabolite, or the interaction between the agent and its target molecule (Manno et al. 2010). Biomarkers of effect are used to verify the interaction of the analyzed substance with the body's receptors, allowing the detection of biological changes, such as chromosomal instability. The frequency of MN in peripheral blood lymphocytes is one of the most used biomarkers to measure genomic damage, mitotic dysfunction, apoptosis, and necrosis in human populations exposed to clastogenic and aneugenic agents (Bonassi et al. 2005). Additionally, susceptibility biomarkers indicate which factors can increase or decrease an individual's risk to respond to an exposure to xenobiotic substances, that is, the genetic variables that condition an organism's response to a stressor. Genetic variations in genes that code for metabolizing enzymes and DNA repair proteins are responsible for the variability of individual response to a given exposure (Manno et al. 2010).

People live exposed to numerous genotoxic agents, the main forms are in their workplace, as industry, agriculture, and production or to environmental pollutants, in the places where they live. Figure 4 summarizes occupational and environmental exposures reviewed in this chapter that are associated with a genotoxic effect through the assessment of MN in peripheral blood lymphocytes.

Fig. 4 Occupational exposure to a range of toxicants can give rise to DNA damage that can be measured by the CBMN-Cyt assay. Current literature highlights increase in MN frequency in individuals occupationally exposed to welding fumes, coal and coal-derivative products, pesticides, formaldehyde, PAHs, and other organic solvents and chemicals specific to laboratory workers, painters, and both textile and shoe factory workers. Created with BioRender

Workers in the welding industry are daily exposed to several compounds harmful to human health such as ultraviolet radiation, toxic metals, and polycyclic aromatic hydrocarbons (PAH). The International Agency for Research on Cancer (IARC) has established that many of the compounds released during welding are considered human carcinogens (Quintana-Sosa et al. 2021). It is estimated that more than ten million people worldwide are occupationally exposed to welding fumes. Several scientific data points to the effects caused on workers exposed to these vapors and their association with DNA damage and cancer risk (Antonini 2003). In a recently published study of welders, the CBMN-Cyt assay was used to assess the effects of this exposure on isolated lymphocytes. Quintana-Sosa et al. (2021) found an increase in MN, NPB, NBUD, and necrotic cells in welders when compared to a control group. According to the authors, the DNA damage induction mechanism that affects welding workers may be due to the activity of free radicals that promote oxidative damage and inflammation.

Other activities that are of interest for their potential to harm human health are those of workers exposed to pesticides. The use of pesticides has grown considerably worldwide, as they are the most effective chemical products in controlling pests, weeds, or plant diseases, which contribute to increasing agricultural productivity. Besides, pesticides have caused great concern due to their mutagenic effects, capable of inducing mutations, DNA damage, and chromosomal alterations (Bolognesi and Holland 2016). There are numerous pesticides with wide distribution and different formulations (including carbamates, organophosphates, pyrethroids, and organochlorines) available on the market; however, the variety of genotoxic compounds arising from the complex mixtures of these formulations are often unknown. When applied together, the genotoxic effects are potentiated, offering greater risks to workers. Some studies are noteworthy for investigating the effects of exposure to complex mixtures of pesticides using genotoxicity biomonitoring tools to assess the mixtures' potential risks (Bolognesi and Holland 2016).

Tobacco (*Nicotiana tabacum*) cultivation is widespread in several countries, and Brazil is considered the second largest tobacco producer in the world. Tobacco production requires a large amount of pesticides, as from the beginning of the tobacco crop season to the harvest time (approximately 10 months), farmers are continuously exposed to these agents and handling of tobacco leaves (Da Silva et al. 2014). Furthermore, when handling the tobacco leaves during harvest, separation, and classification of the leaves, tobacco farmers are also at risk of acute poisoning by nicotine, an alkaloid found in the plant. Acute exposure to nicotine can cause green tobacco sickness (GTS), the green tobacco leaf disease (Arcury et al. 2003). Kahl et al. (2018) evaluated the potential effect of DNA damage in samples from tobacco growers exposed to mixtures of pesticides and nicotine in tobacco crops. Increased DNA damage was observed through the comet assay, associated with the exposure time, and, through the CBMN-Cyt test in lymphocytes, authors found an increase in the frequencies of MN, BN, and BN with NBUD and/or with NPB (Kahl et al. 2018). Children exposed to pesticides (residents in areas of intense pesticide application) also suffer adverse effects. Kapka-Skrzypczak et al. (2019) evaluated 108 Polish children who lived in areas dedicated to agriculture and observed an increase in the

level of DNA strand breaks and an increase in MN in lymphocytes isolated from peripheral blood. Some factors contribute to children being vulnerable to exposure to pesticides, such as developmental, dietary, and physiological factors (Kapka-Skrzypczak et al. 2019). The exploratory behavior of this age group makes them more susceptible to exposure due to contact with pesticides that may occur through ingestion, inhalation, or dermal contact (Roberts et al. 2012). Furthermore, studies suggest that there may be an association between parental pesticide use in their occupational activity and adverse outcomes in infants, such as reduced intrauterine growth, fetal death, premature birth, and congenital anomalies (Longnecker et al. 2001).

Coal industry workers are another class of individuals who carry out activities with potential risk to health and the environment. During coal mining activities, large amounts of coal dust, ash, PAHs, and heavy metals are released into the environment, forming a complex mixture. The complex composition of coal dust can cause an inflammatory process in chronically exposed workers, leading to the development of various diseases, such as pneumoconiosis, progressive massive fibrosis, asbestosis, silicosis, bronchitis, loss of lung function, emphysema, and even stomach, liver, and lung cancer (De Souza et al. 2019). Several studies have been carried out on the effects of exposure to mixtures of coal and residues derived from its combustion. Rohr et al. (2013) verified the genetic damage caused by daily exposure to coal dust in open-pit mine workers in Candiota – Brazil and observed an increase in DNA damage. Authors also observed increased MN and NPB in lymphocytes, in addition to increased frequency of MN in oral mucosa cells (Rohr et al. 2013). Another region with large natural coal reserves is located in the southeast of the department of Guajira, known as El Cerrejón, Colombia's largest open-pit mine. León-Mejía et al. (2011) observed genotoxic effects in open-pit coal mine workers. Lymphocytes were used to perform both comet and MN assay, with observed increase in damage index in the first and increase in MN in the later. The damage observed by the authors results from the mixture present in the coal dust, which has synergistic, additive, and potentiating effects that can interact with cellular mechanisms related to the production of reactive oxygen species (ROS), causing damage to important macromolecules such as DNA, lipids, and proteins (León-Mejía et al. 2011; Rohr et al. 2013).

In addition to the effects observed on coal mine workers, the population living around these mines and other industrial facilities are also environmentally affected. A recent study evaluating the population's environmental exposure to mining activities was performed by Espitia-Pérez et al. (2018). The authors observed an increase in MN frequency in BN cells of individuals living in proximity to the open-pit coal mine compared to unexposed controls, besides a highly significant correlation between particle matters ($PM_{2.5}$) levels and MN frequency in BN cells. The effects observed in this study may be related to the high concentrations of toxic elements, such as metals present in $PM_{2.5}$, generated in mining activities (Espitia-Pérez et al. 2018). Similar results were found by Lemos et al. (2020), in a children population exposed to a mixture of contaminants from different origins, in areas influenced by a petrochemical complex. The population evaluated by authors are in the first wind direction of a petrochemical complex. MN frequencies in peripheral blood samples

collected from children were significantly higher than an external reference site. Moreover, chemical analysis of the organic PM extract from the areas showed the presence of 14 out of the 16 priority PAHs, 8 of them considered carcinogenic to humans (Lemos et al. 2020). Studies point to PM as an important indicator of air pollution and the complex mixture present in PM is a significant risk factor for human health and incidence of neurological, pulmonary, and cardiovascular diseases. Sordo et al. (2019) associated the increase of MN in samples of maternal and umbilical cord blood (UCB) lymphocytes with elevated levels of coarse (PM_{10}) and fine ($PM_{2.5}$) fractions, reported in the Mexico City metropolitan area. One of the hypotheses suggested by the authors is that ultrafine particles might cross the placental barrier causing DNA damage in fetal cells, which may contribute to the development of diseases in childhood or adult life (Sordo et al. 2019).

Other important studies were carried out using MN assay in peripheral blood to assess populations occupationally and environmentally affected by contaminants with genotoxic potential. The review by Annangi et al. (2016) compiled biomonitoring studies focused on individuals occupationally and environmentally exposed to nickel, chromium, arsenic, vanadium, and complex mixtures of metals. Arsenic was the element that deserved the most attention in this review as it was cited in most biomonitoring studies with a potential genotoxic effect in the populations analyzed, due to the increase in MN among exposed individuals. Furthermore, the authors found that studies evaluating complex mixtures using MN assay in lymphocytes were able to find associations with the level of MN frequencies in BN cells and other markers. These results confirm the potential carcinogenic risk associated with metals exposure. In addition to the use of appropriate biomarkers to identify risks, the characterization of the exposure scenario is also essential to obtain conclusive results regarding the effects observed in biomonitoring studies. This information was not clearly found in some studies in the review, which impaired data correlations (reviewed by Annangi et al. 2016).

Due to its economic importance and diversified use, occupational and environmental exposure to formaldehyde (FAL) is also of great concern regarding the effects that this exposure can cause to human health. The IARC classifies FAL as group 1, i.e., a carcinogen product (IARC 2006). A systematic review was performed by Fenech et al. (2016), identifying studies that used CBMN-Cyt assay to measure the genotoxic effects of human exposure to FAL. Seventeen publications were found in this review that evaluated occupational and/or laboratory exposures in the biomedical pathology or research setting and also workers from resin and plywood manufacture exposure to FAL. Thirteen publications were highlighted for observing increases in lymphocyte MN frequency, in individuals exposed to FAL relative to controls (reviewed by Fenech et al. 2016). Furthermore, the authors found a strong positive correlation between the frequency of MN in lymphocytes and the duration of exposure to FAL, which reinforces the potential of chronic exposure to this product to cause cumulative genomic instability (Fenech et al. 2016).

Studies of the effects of exposure to PAHs on human lymphocytes at different occupational exposures were compiled in a systematic review by Sram et al. (2016). Authors analyzed 34 studies that used CBMN-Cyt assays to assess MN induction in

lymphocytes of coke oven workers, aluminum industry, rubber factory, road construction, airport workers, and diesel exposed workers, as well as on environmental exposed groups such as police, volunteers, and children. Twenty-four studies observed an increase in the frequency of micronuclei of these individuals in relation to the control group. It is known that exposure to complex PAH mixtures and the interactions between different constituents can affect the carcinogenic response. Furthermore, the authors observed three important points that confirmed previous findings: the frequency of MN measured using CBMN assay is not significantly affected by smoking, frequency of MN increases with age, and females are more sensitive to PAHs than males (Sram et al. 2016).

Other workers are occupationally exposed to a variety of organic solvents in a complex mixture with other agents, such as paints and metals used by painters, hairdressers, textile industry workers, printers, shoe factories, and laboratories (da Silva 2016). These human populations exposures have been monitored for decades through molecular epidemiological approaches that involve different areas of action, with the intention of reducing damage and assisting in the early detection of negative effects. The biomarkers used to assess the occupational and environmental exposures of the populations are capable of detecting some effect of time and dose response. Furthermore, there is a need to create measures to protect these individuals in order to reduce the risks to human and environmental health.

Micronuclei in Lymphocytes in the Clinical Settings

The use of the toxicological genetics approach is an important tool in the clinic for the search and implementation of biomarkers to identify diseases' early manifestations. Several studies make use of MN assay in lymphocytes for the diagnosis of different pathologies, as well as to identify associations found within the different degrees of severity of manifestations of these pathologies (Fenech et al. 2020). Specifically, the MN assay in lymphocytes is the preferred method in human biomonitoring studies to detect cytogenetic effects and, therefore, it can be highly useful in the clinics. Several studies have demonstrated strong associations between MN increase in lymphocytes and different human pathologies, including reproductive difficulties, birth and developmental defects, neurodegenerative diseases, chronic obstructive and pulmonary diseases (COPD), metabolic disorders, autoimmune diseases (AUD), and some cancers (Fig. 5).

One of the main examples of clinical manifestations associated with MN frequency is aging, which is a natural process that progressively compromises the organism functioning (Fenech and Bonassi 2011). This is a response to cellular senescence, a process that occurs in somatic cells due to telomere shortening and reduced stress responsiveness, increasing homeostatic imbalance (López-Otín et al. 2013). Therefore, there is an increase in genetic damage observed in elderly population when compared to younger individuals (Fenech and Bonassi 2011; López-Otín et al. 2013). It has been shown that MN increase twofold from early years in life (up to 10 years old) to 70 years or above for males. That increase is at least 2.8-fold

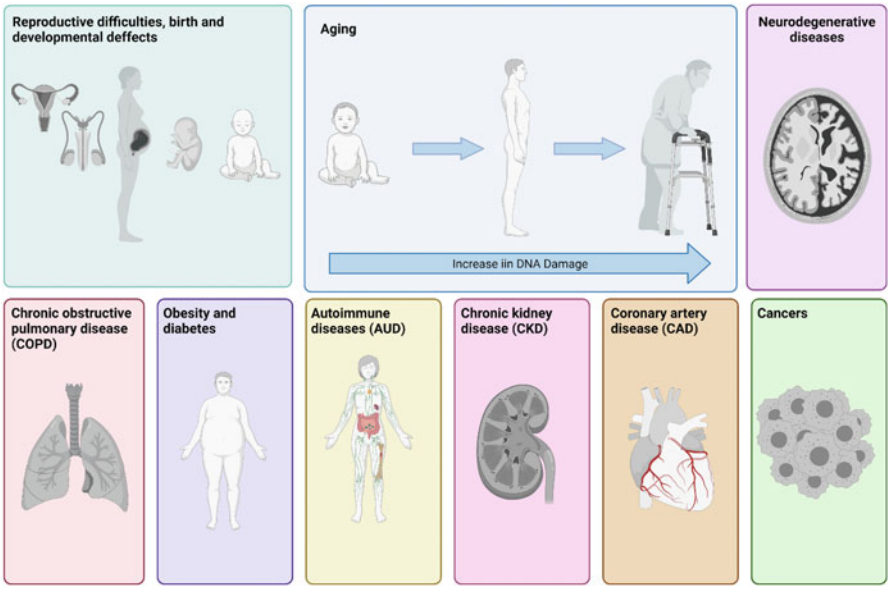

Fig. 5 The MN assay has been shown to be an important tool in clinical settings for both to help to predict or assist in the diagnosis and prognosis of several human conditions, such as reproductive difficulties, birth and developmental defects, aging, neurodegenerative diseases, chronic obstructive pulmonary disease, obesity and diabetes, autoimmune, chronic kidney, and coronary artery diseases, and to a large range of cancers. Created with BioRender

for females, also indicating a gender influence (Fenech and Bonassi 2011). Beyond the natural increase of genomic instability, there is a backlog of DNA damage over the years in humans caused by different exposure types throughout life (López-Otín et al. 2013).

In 2010, Furness et al. (2010) identified that, in a cohort of women of low- and high-risk pregnancies, the presence of MN in lymphocytes was significantly increased in those who developed preeclampsia and/or intrauterine growth restriction before the clinical signs or symptoms appeared. Although this study was the first prospective one investigating the association of chromosomal damage in mid-pregnancy (20 weeks) and pregnancy outcomes, no other research group has explored this aspect (Furness et al. 2010). On the other hand, in a recent review, Knudsen and Kirsch-Volders (2021) pointed out two studies indicating infertility in both men and women with increased MN in relation to their control counterparts. The small sample size in studies points to a gap in the literature and clinical settings for these associations.

The MN assay is well suited to investigate birth and developmental defects since they are often caused by structural or numerical chromosomal aberrations. A Brazilian group analyzed genomic instability biomarkers in children with non-syndromic orofacial clefts and/or palate (NSCL/P). Patients had not only a higher baseline frequency of MN, NBUD, and NPB than the control group, but

also new nuclear morphologies (fused, circular, and horseshoe nuclei) (Xavier et al. 2017). These data indicate that chronic acid folic deficiency is possibly interfering with children's genome instability, which is in accordance with other common foetal malformation, i.e., neural tube defects (NTD) (Bonassi and Fenech 2019). There is only one study available regarding genomic instability in autistic children, measured using the CBMN-Cyt assay, demonstrating a lack of evidence linking both aspects (Main et al. 2015). Down syndrome (DS) is one of the most common developmental defects worldwide, caused by trisomy of chromosome 21. DS patients and their parents (either mother or father or both) have significantly higher frequency of lymphocyte MN, most likely due to a higher rate of chromosome 21 malsegregation during mitosis (reviewed by Bonassi and Fenech 2019).

Neurodegenerative diseases are associated with cellular aging. Thus, the occurrence of chromosomal abnormalities among patients facing neurodegenerative diseases have been investigated (Migliore et al. 2011; Lee et al. 2015; Bonassi and Fenech 2019). The most striking evidence in this field comes from Alzheimer's disease (AD) studies, which indicate increased MN frequency in AD and in Parkinson's disease (PD) patients as well (Petrozzi et al. 2002; Migliore et al. 2011) in blood lymphocytes. A 2.15-fold increase in MN in relation to the control group was observed for AD patients, while for PD patients, the increase was 2.05-fold (Petrozzi et al. 2002). Interestingly, Migliore et al. (2011) and Petrozzi et al. (2002) showed that the chromosomal aberrations in AD arise from whole chromosome malsegregations, while in PD it arises from chromosome breakages. Using the CBMN-Cyt- assay, Lee et al. (2015) identified increased frequency of NBUD in patients with mild cognitive impairment (MCI) when compared to an age- and sex-matched control group. MCI is the precursor stage of AD and is a critical phase to identify potential biomarkers of AD risk and progress monitoring (Lee et al. 2015). Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease with unknown etiology. MN can be found in the brain and spinal cord of ALS patients and their cultured cells (Droppelmann et al. 2019). Increased frequency of MN in cultured lymphocytes from patients in respect to controls have also been shown for patients with ataxia telangiectasia (AT), Werner's syndrome (WS), and Cockayne's syndrome (CS) (Migliore et al. 2011). AT, WS, and CS are characterized by clinical features of physiological aging at an early age, neurological dysfunctions and often neurodegenerative diseases. Further studies that include a larger number of patients in different clinical stages of the pathologies are required.

Chronic obstructive pulmonary disease (COPD) is characterized by irreversible lung damage resulting in airflow limitation, abnormal permanent air-space enlargement, and emphysema. A recent review and meta-analysis found a total of five studies comparing MN frequency in patients affected by COPD versus healthy controls (Asanov et al. 2021). Only two out of the five studies were performed in lymphocytes (Maluf et al. 2007; Najafzadeh et al. 2016). In both, MN frequencies were significantly higher in COPD patients than in controls. The review suggests a circle of events linking inflammation-induced oxidative stress to the risk of COPD through genomic instability and hypoxia. Increased DNA damage, identified by the comet assay, has been shown in patients following pulmonary recovery treatment,

mainly for nonresponders patients and in the ones receiving oxygen therapy (reviewed by Asanov et al. 2021). Although this indicates an integration between clinical parameters with genomic instability, such data has not been explored using the MN in lymphocytes assay, which would provide further insight on permanent DNA damages and their association with clinical parameters and/or intervention.

Obesity and diabetes are metabolic diseases considered a serious and growing health care burden worldwide. A recent review from Franzke et al. (2020) aimed to collect and analyze the data on diabetic, overweight, and obese individuals and their link to chromosomal abnormalities measured by CBMN-Cyt. They found four cross-sectional studies conducted with overweight and obese individuals, including one with children. One study observed increased MN, NPB, and NBUD in lymphocytes of obese participants, but not the ones who were overweight, when compared to a control group (Donmez-Altuntas et al. 2014). The study conducted by Santovito and Gendusa (2020) investigated only overweight and normal weight (i.e., control group) people, and also found an increase in the three parameters (Santovito and Gendusa 2020). Interestingly, the largest cross-sectional study, by Li et al. (2015) with over 700 individuals, showed no significant differences between control group and obese individuals, but a decrease in MN frequencies for overweight people compared to controls (Li et al. 2015).

Twelve studies were conducted with diabetic patients, according to the systematic review by Franzke et al. (2020), two cross-sectional and ten case controls. No differences between controls and diabetic patients were observed in the cross-sectional works. Among the case-control studies, seven were conducted in lymphocytes. Increased MN in patients with type 2 diabetes (T2D) in relation to controls was observed in two studies (Martínez-Pérez et al. 2007; Palazzo et al. 2012), while two other studies found increased frequencies of not only MN, but also NPB and NBUD among T2D patients when compared to controls (Prasad et al. 2015; Salimi et al. 2016). Type 2 diabetes disease progression is linked to further elevated genomic instability (Franzke et al. 2020). A few authors have also investigated dietary interventions in T2D patients, and no differences were observed in any interventional studies using lymphocyte MN assay (Wagner et al. 2021).

DNA sensors from human cells are able to detect self-DNA that leaks into the cytoplasm from disrupted MN. When this disruption happens, the sensing of the leaked DNA induces the cGAS-STING pathway triggering an innate autoimmune response and chronic inflammation. Notably, inflammation can induce MN and vice versa, leading to an inflammation-oxidative DNA damage-MN formation-chromothripsis cycle. Although that places MN in the spotlight of autoimmune diseases (AUD) induction, there are only a few studies assessing MN frequencies in AUD (Kirsch-Volders et al. 2020). From a biomonitoring perspective, a couple of studies observed increase in MN frequencies in lymphocytes of patients with multiple sclerosis, systemic sclerosis, Raynaud's phenomenon associated to autoimmune condition, rheumatoid arthritis, and in autoimmune and nonautoimmune thyroid patients (reviewed by Kirsch-Volders et al. 2020). From a pure clinical perspective, a study found increased levels of MN in patients with vitiligo (Donmez-Altuntas et al. 2008), while three studies found similar results in patients

with Bechet's disease (Hamurcu et al. 2005; Karaman et al. 2009; Binici et al. 2013). A common feature of all abovementioned AUD is increase in inflammation in different tissue types.

Chronic kidney disease (CKD) is distinguished from acute kidney injury when abnormality in kidney structure or function persists for more than 3 months. The CKD etiology should be evaluated by a patient's clinical history, physical examination, and urinary findings where the cause may be due systemic alterations, urinary obstruction, exposure to potential nephrotoxins, history of nephrolithiasis or recurrent urinary tract infections, presence of comorbidities, and family history of kidney disease. These patients might have increased oxidative stress, chronic inflammation, DNA damage, and cancer risk. For this, different markers of genomic damage have been often measured in CKD to analyze its potential in the clinical outcomes prediction. MN frequency was increased in peripheral blood lymphocytes in CKD patients with pre-dialysis or hemodialysis interventions (Stopper et al. 2020). In children, cytogenetic effects in individuals with CKD in pre-dialysis stage on regular hemodialysis and after kidney transplantation were investigated using CBMN-Cyt assay. The authors found increased MN frequency in these children and associations between cytogenetic parameters and creatinine or blood urea nitrogen (Cakmak Demircigil et al. 2011). In their recent review about MN frequency in CKD patients, Stopper et al. (2020) observed that genetic damage seems to increase with the disease stage during the pre-dialysis phase and that different strategies, including supplementation with vitamins or antioxidants and dialysis regimens improvement, can reduce the levels of damage.

Coronary artery disease (CAD) is characterized by the reduction of blood flow to the heart muscle, due to obstruction of major blood vessels that supply the heart, mainly due to atherosclerotic plaque formation. Therefore, CAD can lead to angina, myocardial infarction, and acute coronary syndromes. After the observation of DNA alterations in CAD patients in some studies, the first work evaluating MN frequencies in this disease was published in 2001. An increase of MN frequencies in CAD patients was observed in comparison to health subjects, and a positive relationship between MN frequencies and the CAD severity was noticed (Botto et al. 2001). The DNA damage in atherosclerosis seems to be caused by oxidative stress that could induce many other risk factors. In a recent systematic review, Andreassi et al. (2021) provided evidence of higher MN frequencies in CAD patients in comparison to health controls, even with only eight studies available.

Cancer is a heterogeneous, multistep, and multifactorial disease that involves genetic and epigenetic alterations, in addition to environmental factors. In 2000, D. Hanahan and R.A. Weinberg described the hallmarks of cancer, six biological acquired capabilities during the multistep development of a tumor: evading growth suppressor, activating invasion and metastasis, inducing angiogenesis, sustained proliferative signaling, resisting cell death, and enabling replicative immortality (Hanahan and Weinberg 2000). The same authors presented a review of it in 2011 and included more four characteristics as hallmarks: reprogramming of cellular energy metabolism, evading immune destruction, tumor-promoting inflammation, and genome instability. The last one is highlighted by the authors as the most

prominent due to the potential to orchestrate the other hallmark capabilities to enable cancer driving (Hanahan and Weinberg 2011).

The first study investigating MN frequency in hematological cancer is from 1981, conducted with patients with acute nonlymphocytic leukemia, in which authors observed that lower MN frequency led to better median survival (reviewed by Bonassi and Fenech 2019). Basal MN frequency was also observed to be higher in patients with different forms of leukemia when compared to basal levels of a healthy control group, but no differences among different leukemias was seen (Donmez-Altuntas et al. 2008). In contrast, Wang et al. (2013) found that MN frequency was higher for patients with acute leukemia than patients with myelodysplastic syndrome and benign blood diseases. Following treatment, the overall response rate of patients with lower MN levels was significantly better than the patients with higher MN frequencies (Wang et al. 2013).

Colorectal cancer (CRC) is the second leading cause of cancer deaths in women and third in men. Three major studies highlighted the critical role of genomic instability, through evaluation of MN in lymphocytes, in this cancer type. The first study pointed to increased MN frequencies in patients with CRC adenocarcinoma or with neoplastic polyps when compared to a control group (Karaman et al. 2008). A second study corroborated the significantly increased levels of MN in CRC patients with a twofold increase in relation to healthy controls. Moreover, authors observed a striking association of MN with the plasma clastogenic activity found in CRC patients (Lombardi et al. 2015). The findings from the last study suggest that, at least for the colon area, neoplasia triggering is more relevant for the uprising of MN since the abnormality was found elevated for patients with CRC and neoplastic polyps, but not with inflammatory bowel disease (Maffei et al. 2014).

Lung cancers are the most diagnosed cancer and the leading cause of cancer deaths worldwide. Risk factors for lung cancer include smoking, air pollution, and exposure to carcinogens, such as PAH, among others. A recent systematic review and meta-analysis identified inflammation-induced oxidative stress by smoking and/or environmental exposures, within a background of genetic susceptibility, as the major player in the pathways associated with lung cancer genomic instability (Asanov et al. 2021). In a case-control study, MN levels were increased for lung cancer patients than healthy controls. Among the patients, MN levels were exacerbated if patients were current or former smokers (Cheng et al. 1996). Lung mesothelioma patients also presented increased MN frequency when compared to patients with lung cancer and benign respiratory diseases and to healthy controls (Bolognesi et al. 2005). El-Zein et al. (2006) subjected lymphocytes from lung cancer patients to the CBMN-Cyt assay and found spontaneous increase in MN, NPB, and NBUD (El-Zein et al. 2006). In other patients' cohorts assessed by the same research group, the frequency of MN and NPB was always increased, demonstrating the usefulness of CBMN-Cyt as a confirmatory diagnosis for lung cancer (reviewed by Asanov et al. 2021).

As head and neck cancers (HNC) affect mainly the oral cavity, pharynx, and larynx, the major surrogate for MN analysis in patients with HNC is buccal cells.

Yet, five studies were conducted in HNC patients using lymphocytes (reviewed by Bolognesi et al. 2021). Among them, four studies observed increased MN frequencies in HNC patients when compared to controls. One of the studies encountered increased MN frequency in HNC patients but also in their first-degree relatives (Burgaz et al. 2011). George et al. (2014) analyzed a large group of patients with squamous cell carcinoma at different mucosal sites, observing a striking higher MN level in patients than in controls, accompanied by increase in NBUD and NPB (George et al. 2014). Moreover, the CBMN-Cyt assay was shown to be the best biological indicator to evaluate genetic damage induced by radiation therapy in a cohort of patients with nasopharyngeal cancer (Bolognesi et al. 2021). Interestingly, HNC tumors harbor telomere dysfunction, leading to exacerbated levels of breakage-fusion-bridge (BFB) cycles from anaphase bridges (Gisselsson et al. 2002).

The essential role of BRCA1 and BRCA2 in homologous recombination DNA repair places the frequency of MN as a potential biomarker for breast cancer. Increase in lymphocytes MN frequencies was observed in breast cancer patients when compared to controls (Varga et al. 2004; Santos et al. 2010). A study in 2014 observed only a nonsignificant increase in baseline and postradiation MN in breast cancer patients (reviewed by Bolognesi et al. 2021). That corroborates with the meta-analyses performed in 2012 by Cardinale et al., in which authors observed increased MN in baseline and in radiation-induced patients, but accompanied by such a high level of heterogeneity, turning results inconclusive (Cardinale et al. 2012). From the available studies, there is evidence that mutations in *BRCA1* and *BRCA2*, SNPs in *SLX4/FANCP* genes, and levels of radiation are the main factors affecting MN frequencies in breast cancer patients. Data from literature also points to CBMN-Cyt being a helpful tool to select women for intensive screening for breast cancer (Bonassi and Fenech 2019).

Another cancer type highly associated with genetic background is skin cancer, which is presented either as melanoma or nonmelanoma. Skin cancer is particularly common in Caucasian populations and linked to dysfunctional XPC DNA damage recognition protein required for global genome repair. Cultured lymphocytes and fibroblasts from patients with familial malignant melanoma show increased MN frequencies compared to both control group and nonfamilial subjects (reviewed by Dhillon et al. 2021). Majority of studies conducted with skin cancer patients highlight the hypersensitivity to DNA damage from UV exposure due to defective xeroderma pigmentosum (XP) gene that encodes for the XPC protein. Those studies indicate different SNPs providing different outcomes when MN frequencies are analyzed in XP skin cancer patients, as much as showing that *XP* homozygotes patients have increased MN levels when compared to control subjects and heterozygotes (reviewed by Dhillon et al. 2021). None of those studies evaluated other biomarkers, such as NPB or NBUD, and the utility of the MN assay for skin cancer risk or prediction is still unclear (Bonassi and Fenech 2019; Dhillon et al. 2021).

A male-specific cancer type, prostate cancer, has a single study available assessing MN in lymphocytes between seven cases and seven controls. Authors

did not find any significant differences between the two groups (reviewed by Bonassi and Fenech 2019). On the other hand, cervical cancer is a female-specific cancer type and although there are several studies available, only one was performed using lymphocytes (Gashi et al. 2018). Pathological cervical smears have increased MN frequencies than normal smears, as per a recent review (Setayesh et al. 2021). HPV infection is the major risk for cervical cancer. The presence of high-risk HPV infection shows a 4.9-fold and a 1.9-fold increase in cervical cells MN in relation to a control group and to a group of women with squamous intraepithelial lesions (SIL) but no HPV infection, respectively (reviewed by Setayesh et al. 2021). Corroborating this, the study of Gashi et al. (2018) showed increased frequencies of MN in lymphocytes, accompanied by increased NBUD and NPB as well, in women with high-grade SIL or with invasive squamous cervical cancer. MN frequency was positively correlated with histological-grade cervical lesions (Gashi et al. 2018).

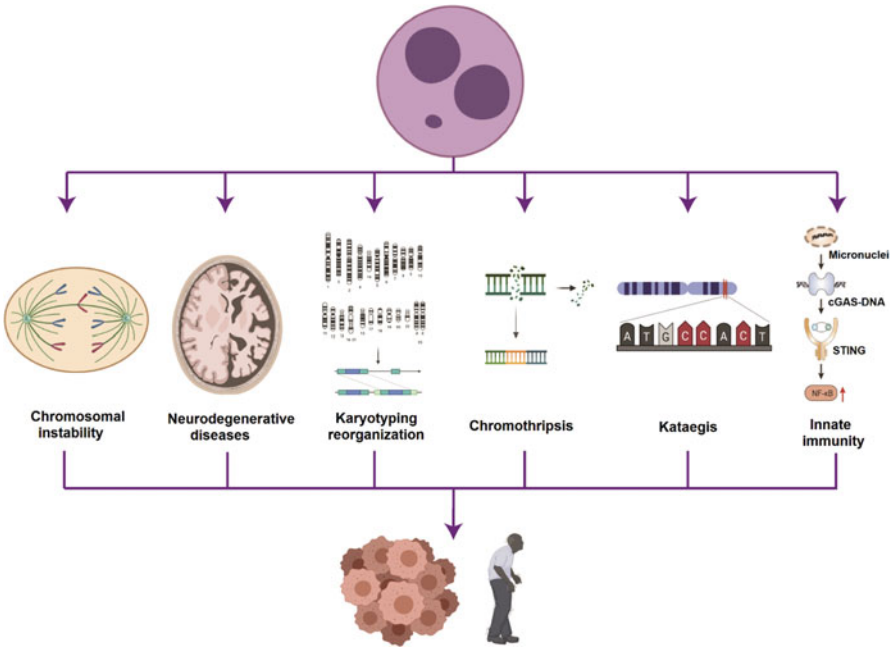

Fig. 6 The reemerging interest in micronuclei studies comes from established mechanisms, such as chromosomal instability and the strong data showing that MN can be potent biomarkers of aging-related diseases, such neurodegenerative ones. New evidence shows that MN are key factors in karyotyping reorganization (i.e., karyotyping code). Moreover, stress response–mediated genomic chaos, arising from events such as chromothripsis and kataegis, are strongly associated with MN. Finally, MN have been shown to play an essential role in the immune system in a non-inflammation manner, and because innate immunity is also triggered by cytosolic DNA, mainly leaked from MN. Importantly, all those events can result in cancer and/or early onset of aging-related diseases. Co-created with BioRender

Applications to Prognosis

Decidedly, the MN test is a great tool to be applied in several areas of environmental exposure and health studies. In recent years, there is an emerging re-interest in the assay (Fig. 6) due to its already established mechanism in giving rise to genomic instability and aging-related diseases, but mainly to new evidence indicating that MN are key factors in karyotyping code, massive DNA damage characterized by chromothripsis and kataegis events, and innate immunity triggering. Hence, the MN test is an effective and safe bioassay to evaluate the impact of the genotoxic agent on lymphocytes and to assist in disease prediction and/or early diagnosis. The limitations observed with the use of the CBMN-Cyt assay in human biomonitoring or as a complementary method in the assessment of genomic instability in relation to different diseases are not referred to the method, but rather to the assessment of human populations. Confounding variables, such as age, lifestyle, and difference in relation to medical treatments, are some factors that lead to heterogeneity of results. It is noticed that a greater effort must be made to obtain larger samples, appropriate control groups, larger control and recording of confounding factors, as well as appropriate statistical analyses.

Key Facts of Micronucleus Assay in Lymphocytes for Human Biomonitoring and Clinical Studies

- The micronucleus test in lymphocyte culture is recommended for monitoring human health.
- Increase in the number of micronuclei is related to genomic instability.
- Human exposure to different environmental and occupational agents shows a relationship with an increase in micronuclei.
- Human diseases that cause genomic instability induce an increase in micronuclei in lymphocytes.
- The use of the micronucleus test with cytochalasin-B in lymphocyte culture allows the assessment of DNA damage and cell death.

Mini Dictionary of Terms

- **Breakage-fusion-bridge.** Also known as the break-joint-bridge cycle, it is a mechanism of chromosomal instability. It occurs when telomeres are shortened or the regions surrounding the telomeres are lost leading to chromatid fusion.
- **Cytokinesis-block micronucleus cytome assay.** A test that can measure DNA damage, cytostatic damage, and cell death in different types of tissues, including lymphocytes. An agent that inhibits cytokinesis during culture (cytochalasin-B) is used to allow the analysis of genomic instability in binucleated cells (damage formed in the last cell division).

- **Micronuclei.** The name given to the small nuclear body that forms when a chromosome or fragment of a chromosome is not incorporated into one of the new nuclei during cell division.
- **Nuclear buds.** Micronucleus-like bodies attached to the nucleus by a fine nucleoplasmic connection. They can be generated similarly to micronuclei during nuclear division or in S phase as an attempt to extrude extra DNA.
- **Nucleoplasmic bridges**. They can originate from dicentric rings and dicentric chromosomes, formed by the joining of their ends to chromosomes.

Summary Points

- Micronuclei are originated when a whole or broken chromosome/chromatin lags at the anaphase of dividing cells and becomes separated from the main chromatin during telophase.
- The micronucleus can be detected in human lymphocytes using a cytostatic agent, and their assessment is named the cytokinesis-block micronucleus cytome (CBMN-Cyt) assay.
- The cytokinesis-block micronucleus cytome assay can be used to assess the genotoxic effect derived from occupational and environmental exposure or as a result of having a chronic disease.
- Micronucleus assay in lymphocytes can be considered an indirect measure of prognosis in clinical studies.
- There is a strong association between the number of micronuclei increases in lymphocytes in individuals exposed to occupational and environmental contaminated areas as well as human diseases.

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Salivary Biomarkers in Toxicology: An Update Narrative

30

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Abstract

Saliva is a complex biofluid, but also a magical biofluid, because it is a mirror of both oral and general health. Multiple general pathologies (of endogenous or exogenous cause) have an effect and expression on the structures of the oral cavity, but also in the excreted biofluids. Oral biofluid is available to practitioners to collect and then evaluate, but not without some challenges related to it: what type of saliva/oral biofluid was collected (stimulated versus unstimulated), at what time of day, what is the relationship with oral hygiene (collected immediately after teeth brushing or at a distance), if there are inflammatory processes in the oral cavity, or what is the salivary flow rate and if it is influenced by certain pathologies or drugs administered. The rate of excretion/diffusion of various compounds (which are intended to be used as salivary biomarkers for detection in toxicology) should also be considered in relation to plasma concentrations and renal excretion.

Keywords

Biomarkers · Toxicity · Saliva · Oral biofluid · Analytical methods · Salivary detection · Noninvasive detection

Abbreviations

AAS	Atomic absorption spectrometry
DAD	Diode array detector
DNA	Deoxyribonucleic acid
DSP	Deliberate self-poisoning
ESI	Electrospray ionization
GC-MS	Gas chromatography–mass spectrometry
HIV	Human immunodeficiency viruses
HPLC-MS/MS	High-performance liquid chromatography with tandem mass spectrometry
HG-AAS	Hydride generation-atomic absorption spectroscopy
ICP-OES	Inductively coupled plasma – optical emission spectroscopy
ICP-MS	Inductively coupled plasma – mass spectrometry
IR	Infrared

IUPAC	International Union of Pure and Applied Chemistry
MALDI	Matrix-assisted laser desorption/ionization
MEKC	Micellar electrokinetic chromatography
NMR	Nuclear magnetic resonance
NSAIDs	Nonsteroidal anti-inflammatory drugs
PCA	Principal component analysis
PLS-DA	Partial least squares discriminant analysis
RNA	Ribonucleic acid
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SERS	Surface-enhanced Raman spectroscopy (SERS)
SPE	Solid phase extraction
TDM	Therapeutic drug monitoring
UHPLC	Ultra high-performance liquid chromatography
UV-Vis	Ultraviolet-visible
WHO	World Health Organization

Introduction

Oral fluid is a mixture of saliva, gingival crevicular fluid, peri-implant crevicular fluid, dentinal tubular fluid, cellular debris, and many other components, and, in recent decades, has attracted the attention of biomedical and forensic researchers as a promising environment for the determination of relevant new markers in these fields. This increasing interest was mainly due to their important attributes, such as minimum- or noninvasive sampling, easy manipulation, low cost, as well as accurate diagnosis of several diseases, pathogens, and illicit substances (Khurshid et al. 2021).

The use of invasive blood testing methods represents nowadays the majority of diagnostic and toxicology tests. Furthermore, invasive tools are also commonly used for routine health monitoring. Thus, the relatively recent introduction of salivary analysis has led to a major paradigm shift in field.

Additionally, the extraordinary development of artificial intelligence and machine learning systems, of concepts based on big data and the application of these concepts in biomedical and toxicological analysis, has allowed the rapid development of oral fluid testing for applications in important fields such as genomics, microbiomics, proteomics, metabolomics, and transcriptomics (Khurshid et al. 2016).

There have been many challenges regarding the use of saliva and oral fluids for diagnostic purposes, as well as in the interpretation of the large amount of data obtained, but despite these problems and obstacles it turned out that the gain is major, especially in terms of the identification of new biomarkers for diagnosing oral and systemic diseases.

Saliva is a clear fluid composed of about 98% water and 2% electrolytes, proteins, glucose, and nitrogenous products like urea and ammonia. Approximately 500–1500 mL of saliva is produced per day by a healthy adult, at a rate of approximately 0.5 mL per minute. On the other hand, several physiological and

pathological conditions such as can quantitatively and qualitatively modify the production of oral fluid. About 65% of unstimulated saliva comes from the submandibular gland, 25% from the parotid gland, 4% from the sublingual gland, and 8% from other minor salivary glands. Under stimulated conditions, the saliva that originates from the parotid gland increases to 50% of total saliva, which can be of importance in saliva sample collection (Gug et al. 2019).

A biomarker can be explained as "a defined characteristic that is measured and evaluated as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention" (Silver and Bethesda 2016). Biomarkers are objective indicators of certain biological states, including pharmacological responses to a therapeutic plan, thus being critical to the rational development of medical therapeutics, serving a variety of purposes, such as screening for early signs of disease in community-based settings, diagnoses confirmations, monitoring effects of treatments, or even the progression of a disease and the prediction of its clinical outcome (Califf 2018).

When blood or urine sampling is not feasible, saliva sampling provides a noninvasive, easy-to-perform alternative. In recent years, developments in scientific and clinical interest designate saliva as a matrix for the analysis of a variety of chemicals. Based on the correlations between blood and salivary levels of different analytes, oral fluid tests have been developed. These newly researched tests can offer greater specificity and sensitivity than traditional sampling methods. Moreover, drugs and metabolites in plasma are distributed to salivary glands within minutes of parenteral administration, passively diffusing into oral fluid. The detection window for drugs in saliva is from less than 1 h to approximately 48 h in other body fluids (Grabenauer et al. 2018).

Factors that influence the passage of drugs from blood to saliva include, first of all, the degree of protein binding. Secondly, another important factor is represented by the blood and oral fluid's pH – a more alkaline pH leads to a reduced drug diffusion. Drugs that are alkaline are generally present at higher concentrations in oral fluid than blood, while acidic drugs are present at lower concentrations (Grabenauer et al. 2018; Hutchinson et al. 2018). More so, the analyte's molecular weight, spatial configuration, and lipid solubility can also influence the molecules' passage from blood to saliva. In addition, the substance's acid dissociation constant, (pK_a), is perhaps the primary determinant that governs the potential utility of saliva therapeutic drug monitoring (TDM) for many drugs (Gug et al. 2019). The significance of pK_a values comes from the importance of the ionization states of drug molecules under physiological conditions. An understanding about a molecule's ionization behavior is essential for accurately predicting and characterizing it (solubility, permeability, absorption, distribution, metabolism, excretion). A drug's pK_a can be calculated by computational methods (Pracht and Grimme 2021). Compounds that have pK_a values lower than 5.5 or higher than 8.5 are not significantly influenced by small changes in salivary pH due to flow rate changes. Accordingly, for neutral, non-protein bound acidic drugs with a high pK_a value, as well as for basic drugs with a low pK_a value, saliva/plasma concentration ratios tend to remain relatively constant (Achparaki et al. 2012; Hutchinson et al. 2018).

The drug molecules are transported from plasma to saliva, across the concentration gradient through lipid membranes, by passive diffusion primarily. Other mechanisms such as ultrafiltration and active transportation through the membrane pores also have a role. Non-bonding to plasma proteins, lipophilia, and neutrality of a drug molecule influence the passive diffusion process. Consequently, high plasma protein binding that prevents passive diffusion through membranes can explain certain drug molecules' low concentrations (Martí-Álamo et al. 2012; Gurusankar et al. 2015; Chatterjee 2019).

This chapter contains a critical and narrative update on the literature regarding the salivary biomarkers and the applications of their detection in toxicology. Furthermore, this study aims to explore the advantages of using different analytical methods in the evaluation of salivary biomarkers.

General Aspects

Biomarkers and Classes of Biomarkers

The biomarkers started to be used in clinical research as well as in clinical practice many years ago and nowadays they are entirely accepted as useful tools in clinical trials. Even though some of biomarkers were well validated in clinical practice such as PSA or CEA, most of them need to be periodically re-evaluated (Strimbu and Tavel 2010).

A biomarker is defined as a "characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions" Several types of biomarkers were defined such as molecular, histologic, radiographic, or physiologic. BEST defines seven biomarker categories: susceptibility/risk, diagnostic, monitoring, prognostic, predictive, pharmacodynamic/response, and safety.

However, a distinction must be made between the "biological biomarker" which refers to a narrow category of medical signs observed from outside the body of a patient and which could be accurately measured in a reproducible way. There are several definitions of biomarkers. The National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Puntmann 2009). The World Health Organization (WHO), in coordination with the United Nations and the International Labor Organization, has defined a biomarker as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" (https://inchem.org/documents/ehc/ehc/ehc222.htm). A more specific definition takes into account the incidence and the outcome of disease, but also the effects of treatments, interventions, and even the environmental exposure, such as to chemicals or nutrients.

Lately, WHO modified the definition of biomarkers and included "almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction" (World Health Organisation (WHO) International Programme on Chemical Safety (IPCS) 1993).

According to their applications, several subtypes of biomarkers have been defined, each with clear distinguishing features. Pharmacodynamic/response biomarkers are useful in both clinical practice and early therapeutic development. For many drugs, dosing is determined by measured change in a biomarker when a therapy is given. Most times, when a new treatment is first prescribed, the change in this type of biomarkers is a measurement of dosing. Pharmacodynamic and toxicodynamic interactions caused by individual and environment-related factors impose a need to implement TDM, with importance in both the efficacy and the safety of the drug therapy (Benjamin Chun-Kit Tong 2019). Furthermore, there are numerous interindividual variability in biomarker baseline levels and quantifying various molecules in biological fluids and tissues remains the primary practice to find novel biomarkers (Veytsman and Baranova 2015).

The role of biomarkers is quite important and refers to the confirmation of a diagnosis, of an acute or chronic poisoning or to assess the treatment effectiveness. In some pathologies the biomarkers are used to evaluate the prognosis.

To be used in diagnosis and prognosis, the selection and the validation of biomarkers require several steps in which their specificity and sensitivity must be evaluated. The WHO recommends several steps in the selection of the biomarkers, especially for the toxicity assessment:

1. The identification and definition of the end-point of interest
2. The assembly of the database to document the relationship between the chemical exposure, the possible biomarkers, and the end-point
3. Selection of biomarker specific to the outcome of interest with careful consideration of the biomarker to identify what is being quantified, to assess the sensitivity and specificity of the marker in relation to exposure, and the significance with respect to health outcome or pathological change over time
4. Consideration of specimens potentially available for analysis, with emphasis on protecting the integrity of the specimen between collection and analysis, and a preference for noninvasive techniques
5. Review of the analytical procedures available for quantification of biomarkers and their limitations with respect to detection limit, sensitivity, precision, and accuracy
6. Establishment of an appropriate analytical protocol with provision for quality assurance and quality control
7. Evaluation of intra- and interindividual variation for a non-exposed population
8. Analysis of the database to establish dose-effect and dose-response relationships and their variation, with special emphasis on susceptible individuals

9. Calculation or prediction of risk to human health either for the general population or a sub-group
10. Review of ethical and social considerations

It is worth to mention that biomarkers play an important role in large trials for important pathologies like cancer and heart diseases but also in clinical research and in drug monitoring. However, they are not clinical endpoints and their validation in diagnosis is still work in progress.

Saliva as a Biological Fluid for the Detection of Markers and Biomarkers for Diagnosis and Toxicology Assessment

One of the most complex body fluids is represented by saliva. This biological fluid can be assimilated with a mirror of the human body since it reflects the homeostasis of the oral cavity but it can also contain important clues about the proper functioning of the whole body or about the existence of a medical condition, even from the incipient phase in some cases. Moreover, saliva is easily collected, in a minimally invasive manner and at low cost because it does not require laborious processing before testing as well as for short- and medium-term storage.

Thus, saliva has become increasingly applied as a biological sample since it contains a rich pool of proteins, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), hormones, growth factors, antibodies, amino acids, enzymes, microorganisms, and other molecules with a metabolic or functional role. These compounds may represent biomarkers, thus being useful for the detection purpose in diagnosis of oral and systemic diseases and treatment monitoring (Gug et al. 2019; Cui et al. 2022; Uchida and Ovitt 2022). The importance of salivary components as biomarkers for the diagnosis of some severe medical conditions such as cardiovascular disease, diabetes, Sjögren's syndrome, hepatocellular carcinoma, leukoplakia, and various cancers has been recently investigated and reported in the literature (Malathi et al. 2014; Ilea et al. 2019).

Unlike other biological fluids such as whole blood, blood derivatives and cerebrospinal fluid samples, or tissue samples, saliva is a biological sample that can be collected in a noninvasive manner and without the need for specialized personnel. Storage and manipulation of saliva samples are much easier compared to the samples mentioned above. Also, using saliva increases patient compliance and reduces the overall cost of the analysis (Gug et al. 2019). One of the disadvantages of using saliva is that the level of biomarkers is usually lower than in blood, therefore, analytical methods with very good sensitivity are needed for the analysis of salivary biomarkers (Ilea et al. 2019).

The use of saliva as a biological sample for the determination of biomarkers, including cytokines, involves performing several steps: patient training, collecting samples, storage, pretreatment of the sample, and only after all these steps the detection can be performed. There are two methods usually applied for collecting

saliva: passive drool and saliva collection with absorbent materials such as cotton swabs. The use of passive drool is preferred because when saliva is collected with absorbent material, a filtration step is required, which can lead to decreased cytokine levels (Riis et al. 2021). Commercially available devices called Salivette are used to collect saliva. These are used after the patients rinse their mouth with water and involves chewing a tampon for a few minutes. The saliva swab is then placed in a sterile vial and centrifuged (Sánchez-Tirado et al. 2020). Usually, saliva is collected in sterile tubes (e.g., polypropylene tubes) and is centrifuged, and the supernatant obtained is immediately stored at low temperature of − 80 °C until use; this step is necessary since many compounds such as enzymes and bacteria are present in saliva (Costantini et al. 2020; Medara et al. 2020). The rate of the oral fluid flow and the samples that stimulate fluid production have a major impact. Samples obtained by saliva stimulation (mechanically by chewing or chemically by the use of citric acid) can usually reduce drug concentration, unlike a non-stimulated collection (e.g., spitting). By increasing the salivary flow, the bicarbonate electrolyte concentration rises, which can modify the pH in becoming more alkaline. This decreases the saliva-to-plasma ratio of weakly basic drug molecules, with pK_a values close to the pH of the saliva (6.0–7.8). Therefore, the saliva collection method must be taken into account when quantitatively interpreting the analysis results (Chatterjee 2019).

Another aspect to be taken into consideration is the preservation and the storage of the saliva samples until analysis. To avoid altering the biological matrix between sampling and storage, saliva is kept at low temperatures, usually on ice. Both the chemical composition of the saliva and the saprophytic microbial flora would be influenced if stored at room temperature (Riis et al. 2021). Saliva can be collected in the presence of substances with a preservative role, such as protease inhibitors. It is also important that the time interval between sampling and storage be as short as possible to avoid degradation (Medara et al. 2020).

It is important to note that saliva may facilitate toxicological and biochemical investigation, being considered the best research tool for scientific investigations on humans from the ethical point of view. In this case, too, the patient's consent is required prior to the collection of samples, and all local guidelines regarding the work with human saliva must be followed.

The applications tested so far for salivary detection refer to the detection of drugs of abuse and alcohol (Mishra et al. 2020), forensic medicine (Mani et al. 2021a), and the correlation between the levels of drugs in saliva and blood (Gug et al. 2019). Saliva has been successfully used for the diagnosis of several diseases, based on the evaluation of several biomarkers contained in this matrix. The envisaged pathologies are oral cancers, diabetes or Cushing's syndrome, Sjögren's syndrome, Hashimoto thyroiditis, celiac disease, human immunodeficiency viruses (HIV), viral hepatitis, malaria, gastroesophageal reflux, Alzheimer's disease, breast, ovaries, pancreas, or lung cancers, as well as for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in the last 2 years, during the pandemic (Gug et al. 2019; Mani et al. 2021a; Ahmadieh et al. 2022).

Toxicology and Biomarkers

Biomarkers involved in pathologies related to toxicological causes can be classified in different ways. One approach would be by classifying them according to the affected functional systems (e.g. digestive system, neurologic system, cardiovascular system). Another approach would be to group them according to their chemical classification. However, Timbrell (1998) proposed a toxicological classification of biomarkers, revealing different types of toxicity-related classes of biomarkers, which we are going to assume forward (Fig. 1):

- Biomarkers of exposure
- Biomarkers of response (or toxic effect)
- Biomarkers of susceptibility

Of course, in some cases, these categories may overlap.

Biomarkers of Exposure

These biomarkers are an indication of occurrence and/or extent of exposure to a certain toxicological molecule.

Many types of salivary biomarkers can be found in this category, as saliva together with the oral mucosa represents a gate from the outside world toward the whole body, which is exposed to different toxicological factors.

A large number of studies (Scherer 2006; Matsumoto et al. 2013; Soni et al. 2016; Jacob et al. 2017; Comiford et al. 2018; Mani et al. 2018; Jacobson et al. 2022) have recently been published concerning the exposure to tobacco and e-cigarette smoke.

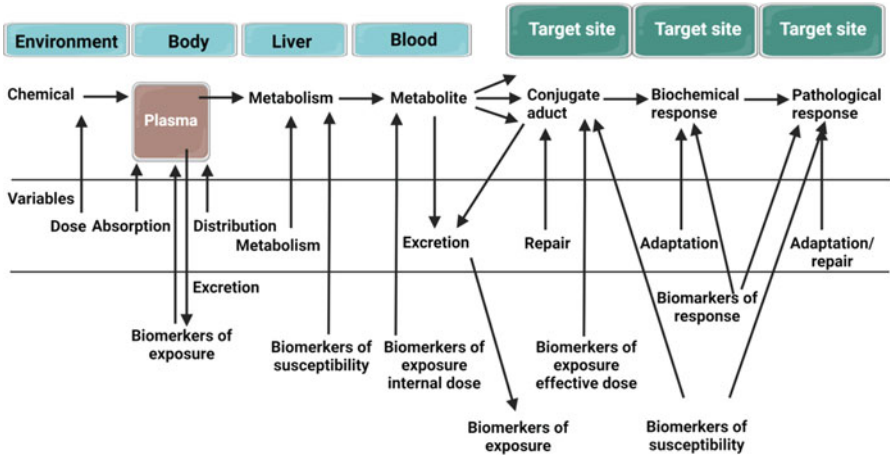

Fig. 1 Biomarkers and the connections between different types of biomarkers. The nature of, and interrelationships between, the three categories of biomarkers and the variables which affect them. (Adapted with permission after (Timbrell 1998))

At the same time, there are studies revealing exposure to different toxic metals (manganese (Mani et al. 2018), lead (Sun et al. 2022), etc.), or illicit drugs (Hutchinson et al. 2018).

Biomarkers of Response or Toxic Effect

These biomarkers reveal the changes that have happened in the body because of the toxic element to which it has been exposed. The biomarkers can be invasive or noninvasive, according to their determination method. In case of salivary biomarkers, there is the advantage of having noninvasive biomarkers, because saliva collection is a noninvasive process. Among salivary biomarkers of response (or toxic effect), there can be found enzyme inhibitions or inductions (Solé et al. 2003), cytokine levels modifications (Jagannath et al. 2020), and other endogenous metabolites modifications (Sánchez-Tirado et al. 2020).

Biomarkers of Susceptibility

These biomarkers reveal how different phenotypes respond to toxic elements. These are mostly related to the metabolism stage and the most common example is the enzymatic polymorphism.

Analytical Methods

Separation Methods

The state-of-the-art methods (Gug et al. 2019) used for the analysis of salivary biomarkers are separation methods, more exactly gas chromatography–mass spectrometry (GC-MS) and high-performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS). They are generally used as validation methods when developing new alternative approaches such as electrochemical biosensing methods (Mani et al. 2021b), immunological methods (Khurshid et al. 2021; Ahmadieh et al. 2022), or chemometric approaches (Colceriu-Şimon et al. 2019).

HPLC-MS/MS (Ashraf et al. 2021) is one of the most utilized analytical methods, offering great versatility, and, in the same time, exhibiting high sensitivity and selectivity. It offers the possibility of using a wide range of solvents in combination with different types of stationary phases (i.e., chromatographic columns), thus giving the possibility of separating almost any analyte. For being very robust, it represents the standard of analysis in many domains, including medical, pharmaceutical, environmental, and toxicological analysis. If using ultra-high-performance liquid chromatography (UHPLC) (Brown et al. 2014; Kämäräinen et al. 2018), this offers lower analysis times (coupled with higher pressures), keeping the great selectivity. Different types of detectors can be coupled with the HPLC system, by which the two

most used are by far diode array detector (DAD) and the MS detector. The DAD is cheaper, it can be used for any substance which absorbs in the ultraviolet-visible (UV-Vis) domain (or can be derivatized to such a molecule) and is very reliable. When using the MS detector, this adds the possibility of uniquely identifying the separated molecules by their mass spectra. However, the coupling HPLC-MS is not the ideal one, and an interface between the separation system and the detector which vaporizes the sample before getting to the MS is needed, thus getting the costs of the analysis higher.

HPLC-MS has been applied for the detection and quantification of salivary biomarkers in case of toxic substance exposure, obtaining limits of detection of 0.07 ng/mL in case of mycophenolic acid (Wiesen et al. 2012), a drug used post-renal transplant.

However, the GC-MS (Duffy and Morrin 2019) represents an ideal coupling, the sample being already vaporized when getting to the MS detector. In GC, the great separation efficacy is also combined with high selectivity and specificity. The latter is given also by the MS detection.

Electrochemical Methods

Electrochemical detection methods are a good and beneficial alternative to conventional methods, solving some of the problems encountered in traditional detection. According to the International Union of Pure and Applied Chemistry (IUPAC) definition, electrochemical sensors are represented by the detection devices that are able of converting the chemical signal into the analytical one (Hulanicki et al. 1991). Thus, electrochemical sensors are characterized by high sensitivity, specificity, short analysis time, low cost, and use of small amounts of sample and solvent. Electrochemical sensors use an electrode as a transducer, which measures the analytical signal generated by the analyte-receptor interaction, which in this case is the working surface of the electrode, modified with various elements to increase the selectivity for a particular analyte. In the case of these sensors, the detection is based on an electrochemical reaction, which results in a change of potential, current, or conductivity (Islam and Channon 2020). Electrochemical analysis involves collecting the biological sample, applying a pretreatment when needed and then using an electrochemical sensor and procedure to obtain qualitative and quantitative information about the analyte of interest.

A large variety of electrochemical techniques are available for the salivary detection of biomarkers which are relevant from medical point of view. Despite all these advantages, electrochemical assays are relatively less common in biomedical and diagnostic applications, since in many cases, the developed assays and the optimized methods have not been tested in biological samples. This is mainly due to the lack of validation related with the difficulty of electrochemical measurement in real samples, to poor specificity as there are potential interferences. If the proposed methods are confirmed by validation, there will be more of these procedures used in diagnostic field (Gug et al. 2019).

Spectral Methods

Spectral methods are the oldest and robust analysis methods used for detecting a plethora of targets including toxicity biomarkers. Optical methods show promising results in detecting and quantifying the toxicity biomarkers taking advantage of the rapidity of the analysis, increased accuracy, and multiplexing. In recent years, the development of portable small instruments requiring small volumes of samples pushed the use of spectral methods from benchtop clinical to bedside use (Hardy et al. 2022).

Besides the UV-Vis and Infrared absorption spectroscopic techniques which were intensively used as benchtop techniques but are limited in sensitivity, new techniques started to be used for quantification of toxicity biomarkers are: Mass Spectrometry (MS), Nuclear Magnetic Resonance (NMR), Raman Spectroscopy with all the derived techniques like Surface Enhanced Raman Spectroscopy (SERS), Resonance Raman Spectroscopy, or Nonlinear Raman spectroscopies. All those techniques are adaptable for analyzing small saliva samples. A synthetic presentation of the spectroscopic methods used in the analysis of salivary biomarkers is presented in Fig. 2 (Hardy et al. 2022).

Mass spectrometry (MS) is able to examine a salivary proteome in minute details giving details related to the presence or absence, level of expression, as well as posttranslational modifications of multiple biomarkers in a salivary proteome theoretically altered by diseases or interventions (Al-Tarawneh et al. 2011). The principle of MS is relatively simply explained as the generation of multiple ions from the sample under investigation and the separation of them according to their specific mass-to-charge ratio (m/z), and then the recording of the relative abundance of each ion type. The development of two ionization techniques, electrospray (ESI) and

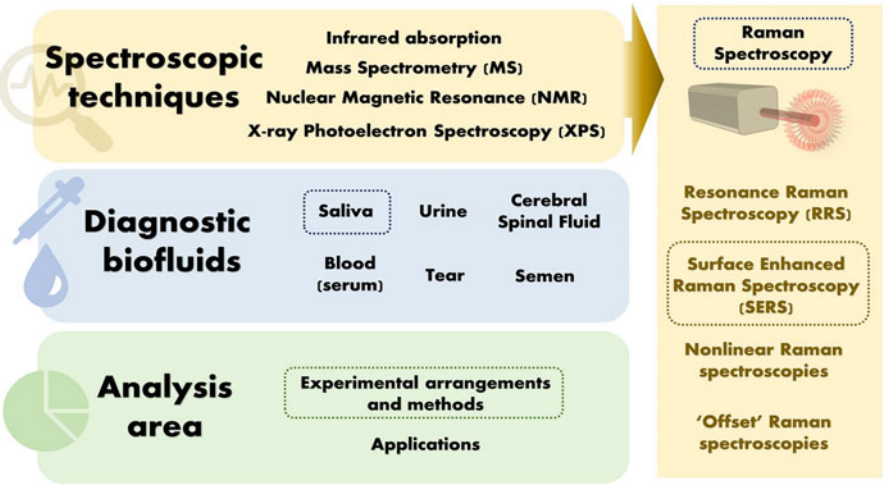

Fig. 2 Classification of spectroscopic techniques. Spectroscopic techniques, diagnostic biofluids, and analysis area. (Reprinted with permission from (Hardy et al. 2022) (open access))

matrix-assisted laser desorption/ionization (MALDI), has revolutionized the applicability of mass spectrometry to almost any biological molecule (Glish and Vachet 2003). Some drawbacks were reported for MS, the technique is relatively slow, requires skilled users, and can suffer from non-universal ionization efficiency and ion suppression (Ma et al. 2020).

Raman Spectroscopy (RS) needs a monochromatic light source, optics to remove unwanted light, and a spectrograph/monochromator to isolate a specific wavelength range. The improved capabilities of cameras and advances in analysis software were advantages that RS profit. The Raman scattering phenomenon relies on the instantaneous inelastic interaction of light with molecular vibrations, whereby a change in bond polarizability as a function of nuclear motions results in an alteration to the emitted frequency of light known as Raman-shift. With RS a "molecular fingerprint" is obtained. After years of application in food, environment, and artwork samples analyses, the studies performed by Berger et al. showed that Raman spectroscopy could be used to analyze biofluids (Berger et al. 1999). Recently, a number of studies proved that RS is a powerful technique for early disease diagnosis (Larmour and Graham 2011; Sharma et al. 2012; Maitra et al. 2020).

Several portable Raman equipment are currently commercially available but, for the moment, they are limited to forensic and environmental analysis (Metrohm®, OceanInsight®, RSDynamics®).

Chemometrics

Chemometrics, as defined by Héberger K. in reference (Vékey et al. 2008), is "the chemical discipline that uses mathematical, statistical, and other methods employing formal logic to design or select optimal measurement procedures and experiments, and to provide maximum relevant chemical information by analyzing chemical data." It practically manipulates the data obtained from chemical analysis and gives it mathematical and statistical interpretation. As consequence, it is a valuable tool used to extract relevant information from big chemical data by applying multivariate analysis and pattern recognition. The statistical interpretation can be employed to chemical data obtained from various analytical methods, but the spectroscopic methods are the most utilized in chemometric analysis (Mani et al. 2018). Usually infrared (IR) and/or Raman spectral data (from different approaches) is statistically interpreted to collect chemometric results.

There are several types of statistical models that can be implemented in the data interpretation. One of the most used models is represented by principal component analysis (PCA) (Jollife and Cadima 2016), which is an exploratory data analysis tool used to build predictive models. It is very versatile and can be applied to the analysis of various types of data. It basically identifies the linear combination with maximum variance between p numerical variables and n entities or individuals. The partial least squares discriminant analysis (PLS-DA) is another predictive tool used in chemometrics and medical diagnosis prediction. It can be applied to any data type, not only to chemical data. It combines features of PCA and multiple regression.

Toxicity and Sources of Toxicity

Pollutant-Induced Toxicity

Metal exposure intoxications represent, unfortunately, quite common health issues and they seem to be increasing as we live in more and more polluted environments. Manganese, lead, or arsenic exposure and their effects have been studied using different analytical techniques.

Eight toxic and nontoxic metal elements (As, Mn, Mo, Ni, Pb, Ba, Zn, Se, Cr, U) (Bhowmick et al. 2015) have been studied by hydride generation-atomic absorption spectroscopy (HG-AAS), in case of As, and by inductively coupled plasma – mass spectrometry (ICP-MS), for the other metals. Statistical interpretation of data has been made using the PCA approach. Statistically significant higher levels of As and Mn in water were found, compared to limits recommended by World Health Organization (WHO). In the same time, these levels were correlated with their levels in saliva, indicating the exposure of subjects to these toxic elements. Moreover, there was a negative correlation between As levels in saliva and Zn and/or Se levels, thus suggesting that Zn and/or Se deficiencies may be mediating As exposure.

Manganese exposure because of its presence in drinking water has been also studied in children's saliva, hair, and toenails (Ntihabose et al. 2018). Results have revealed significant correlation between Mn levels in biological samples (saliva supernatant, hair, toenails) and Mn levels in drinking water. However, the correlation in case of saliva was weak, but the use of hair or toenails stays promising for using them for the determination of long-term exposure at even low levels of Mn.

Different types of radiation exposure can influence the body's response in various ways. It is well known that exposure to UV (of type A, B or C) radiation can lead to different types of cancer, among which melanoma is the most frequently diagnosed. Soni et al. (2016) have studied the effects of salivary biomarker modification in animals, after being treated with cobalt-60 gamma radiation. Results have shown a significant decrease in sodium salivary levels, as well as a significant increase in levels of potassium, urea, total protein, and amylase in saliva of studied animals, even at low doses of radiation. At the same time, exposure to low dose of ionizing radiation can be associated with changes in levels of thiocyanate salivary ion, as results of a chemometric surface-enhanced Raman spectroscopy (SERS) study have shown (Colceriu-Șimon et al. 2019).

Toxicity Induced by Local Pathologies

Authors (Herman et al. 2016) have studied by ICP-MS and inductively coupled plasma – optical emission spectroscopy (ICP-OES) the correlation between eight essential and toxic metals (Ca, Cu, Fe, Mg, Mn, Zn, Fe, Pb) present in saliva and

periodontal disease. In all cases, higher levels of metals were found, but only three of them showed significant correlation with the presence of periodontal disease: Cu, Mg, and Mn ($p < 0.05$). In these cases, the levels of salivary metal were two to five times higher in periodontal patients than in healthy controls.

Toxicity Induced by Drug Screening

Drug toxicity has been studied since long time, for almost all classes of medical compounds. As it is known, a certain molecule can have a beneficial effect if it is administered up to a level and, usually, if this level is overpassed, the molecule becomes toxic.

For example, Wiesen et al. developed a simple HPLC-MS/MS method (Wiesen et al. 2012) for the determination of mycophenolic acid and its glucuronide in saliva and plasma. The two molecules are used as metabolites in drug screening for patients post-renal transplant. The developed method can be applied even to children saliva, which comes with the advantage of being a noninvasive biological sample, thus increasing the patient compliance.

The comparative analysis of several analgesics and anti-epileptic drugs from salivary samples are presented in Tables 1 and 2, respectively.

Toxicity Induced by Tobacco and Drugs of Abuse

Tobacco products exposure has been studied for over 30 years (Etzel 1990). Since then, there are some molecules that have been generally recognized as biomarkers of exposure to tobacco products and tobacco smoke (Mani et al. 2018):

- Nicotine and its main metabolite, cotinine
- SCN^- (thiocyanate)
- CO (carbon monoxide)
- Tobacco-specific nitrosamines
- Metals

Authors (Matsumoto et al. 2013) have realized a tobacco smoke topography and analyzed nicotine and cotinine levels not only in saliva, as biomarkers of exposure, but also in the mainstream smoke, which was generated by a smoke machine, using two protocols. When using the ISO (International Standardization Organization) protocol, the levels of nicotine and cotinine were found in the accepted ranges as declared by producers ("ultra-low," "low," "medium," "high"). However, when using the HCI (Health Canada Intense) protocol for generating the mainstream smoke, the researchers have found levels of nicotine and cotinine of 1.9- to 5.0-folds higher. Similar results were obtained with tar and CO.

Table 1 Analysis of several analgesics from salivary samples

Drug name/ class	Salivary detection method	Main results	Other mentions	Ref.
Acetaminophen (paracetamol)	HPLC-MS/ MS	Salivary and plasma levels correlated	Hepatic toxicity Used in DSP N-acetyl-cysteine as antidote	Wade et al. (2008))
		Lower limit of detection and its stability are all similar to the plasma samples		Ryan et al. (2009))
	AAS	Analysis of paracetamol in its pharmaceutical formulations, without any interference from other excipients	Therapeutic level 10–25 μg/mL, toxic level > 200 μg/mL	Issa et al. (2008))
Non-steroidal anti-inflammatory drugs (NSAIDs)	SPE + MEKC	Salivary concentrations detected above 0.1 μg/L and provided a linear relationship between peak area and the concentrations of NSAIDs ($R^2 > 0.9994$)		Almeda et al. (2008)
	HPLC-ESI-MS	Satisfactory recoveries (101.3–106.1%), achieving saliva-based noninvasive pharmacokinetic analyses of ibuprofen enantiomers		Ogawa et al. (2014)
		Linear calibration curve with $R^2 = 0.9997$		Pochivalov et al. (2017)
	LLE	Good linearity with $R^2 = 0.9955$–0.9998 and 0.9–7.7% relative standard deviations		Hassan and Alshana (2019)
	HPLC-DAD	95.7–109.2% for recoveries Linearity ($R^2 \geq 0.9821$) from 0.08 to 8.0 μg/mL		Tartaglia et al. (2020)
Opioids	GC-MS	Correlation coefficient for the calibration curves of $R^2 = 0.9977$ for codeine		Cámpora et al. (2006)
	LC-MS	300 oral fluid specimens tested		Cone et al. (2015)
		Correlation coefficients of $R^2 = 0.999$ for hydrocodone and norhydrocodone and $R^2 = 0.998$ for hydromorphone, with determination accuracy over 92.3%		Tuyay et al. (2012)
	Lateral flow assay	Determined artificially added codeine to saliva From 0.5 to 5 μg/mL in 5 min		Shende et al. (2019)

Table 2 Analysis of several anti-epileptic drugs from salivary samples

Drug name/class	Salivary detection method	Main results	Other mentions	Ref.
Valproic acid (valproates)	LC-MS	Mean recovery of 99.4%, and $R^2 = 0.9989$ for valproate	Plasma protein-bound is concentration dependent, with variations from 74 to 93%	Patsalos et al. (2018, 2017)
		Mean recovery of 50.6 ± 6.5 from dried saliva spots		Tonic-Ribarska et al. (2012), Patsalos et al. (2017)
		A good linear relationship between the salivary and the serum-free valproic acid		Dwivedi et al. (2015), Tonic-Ribarska et al. (2012)
Benzodiazepines	LC-MS	Salivary concentrations are highly correlated with serum concentrations ($R^2 = 0.93$ and $R^2 = 0.90$) for clobazam	Clobazam excessive accumulation correlated with toxicity	Patsalos et al. (2018), Tonic-Ribarska et al. (2012)
		A correlation coefficient of above 0.99 for several tested drugs (including benzodiazepines)		Concheiro et al. (2008)
		Tests performed on over 2000 people, where in oral fluid samples, the mean sensitivity, specificity, and accuracy were reportedly 74.4%, 84.2%, and 79.2%, while for whole blood samples these mean percentages were 66.7%, 87.0%, and, 74.4%, respectively		Pil and Verstraete (2008)
	GC-MS	Clonazepam with a 72.8% recovery and a 0.992 correlation coefficient Midazolam with a 73.1% recovery and an $R^2 = 0.997$ correlation coefficient		Gunnar et al. (2005)
Carbamazepine	HPLC-UV	Strong correlation between blood and	Drug-drug interactions in CYP	Patsalos et al. (2018),

(continued)

Table 2 (continued)

Drug name/class	Salivary detection method	Main results	Other mentions	Ref.
		saliva samples, $R^2 = 0.76$-0.88 In patients with acute poisonings, consequently to different ingested doses of carbamazepine, the authors noted high interindividual variations, with a strong correlation between saliva and serum levels ($R^2 = 0.9117$)	metabolism (inhibitions, inductions) Simultaneously taking carbamazepine and lamotrigine may increase the prospect of neurotoxic side effects Narrow effective range, patient interindividuality	Djordjevic et al. (2009)
Phenobarbital	LC-DAD	Oral fluid phenobarbital concentrations correlate with blood phenobarbital concentrations at values of $R^2 = 0.64$–0.98 for total phenobarbital and $R^2 = 0.64$–0.99 for free phenobarbital	Drug-drug interactions in CYP metabolism (inhibitions, inductions)	Patsalos and Berry (2013), Patsalos et al. (2018), Patrick et al. (2021)

Influence of Toxic Factors on Biomarker Levels

Humans are daily exposed to toxic environmental agents, some of which have gradually been elucidated to be known as important risk factors for several diseases. Inhaled particles, heavy metals, organohalogen compounds, pesticides and plasticizers are among the common environmental exposures in humans. These compounds can enter the human body through diet, water, air, or direct skin contact. From early childhood or even in utero, toxicant exposures can alter gene expression, as well as cellular and other physiological processes. Studies have reported that exposure to multiple air pollutants in the prenatal period and infancy can lead to a decrease in psychomotor development and cognitive performance, decreased adaptive functioning, and decreased behavioral indices. With an increase in the variability in man-made chemicals during the past decades, the risk assessment of total exposure is more challenging and, thus, more necessary than ever (Jacobson et al. 2022; Malecki et al. 2022; Sun et al. 2022).

The exposome concept was put forward in 2005 by Christopher Wild to complement the genome concept that characterizes the total exposure of an individual to environmental and lifestyle factors. Hence, the exposome concept can define the impact that environmental exposure has on human health and can contribute to its

further understanding in the context of how people biologically respond to it. Moreover, this research paradigm is necessary in order to establish a clear causality and to further characterize these toxic factors' impact on health. However, further research is needed in order to fully understand the complex chemical mixtures that public health is facing (Wild 2005; Sun et al. 2022).

With environmental chemicals' potential to cause diverse DNA damage, biomarkers of exposure provide information regarding the exposure dose and its effects, with relevance in the risk assessment process. This type of biomarker can be assessed in biological fluids samples such as saliva. For people living in areas with contaminated air, biomonitoring of pollutants should be a high priority. Also, biomarkers of susceptibility, of genotoxicity, and of oxidative stress can determine other modifications in the body that are derived from exposure to potentially toxic agents. All these types of biomarkers depend on the type, degree, and time of environmental xenobiotics exposure and can help improve the evaluation of health risks. Metabolomics can be used to identify potential biomarkers and their biological mechanisms in areas such as environmental exposure assessment, providing a better understanding of those pollutants' toxic effects (Wei et al. 2022). The primary mechanism by which xenobiotics leave the blood and enter the saliva is passive diffusion. Therefore, salivary determinations can be used to accurately estimate systemic analyte doses in scenarios where oral fluid concentrations are correlated with blood concentrations.

Some environmental chemicals can impact the metabolome and overall health by inducing dysbiosis in the microbiome The gut microbiome is noted to be prone to the effects of mixed xenobiotics, acting as a health mediator of environmental stressors, toxicant mixtures, and neurobiology (Turesky and Lu 2020; Sun et al. 2022). It is noted that there are connections between gut dysbiosis and the development of numerous age-related diseases (Malecki et al. 2022).

Biological aging is closely related to environmental toxicity. Environmental toxic exposures can induce systemic inflammation and oxidative stress that can impact the aging processes. A diminished ability to metabolize, compensate, and recover from exposure to adverse stress agents can alter the homeostasis of biological systems. Not to mention that an accumulation of different types of biological and environmental factors across the life span can increase age-related biological susceptibility. Therefore, an exacerbation of cognitive impairment, as well as chronic cardiometabolic and respiratory diseases is likely, with an impact on neurological and cognitive functions (Malecki et al. 2022).

Different environmental toxicants induce different metabolic effects (Sun et al. 2022):

- Heavy metal ions can enter the human body and successively translocate to different tissues, where they can accumulate and develop distinct pathologies. Without characteristic symptoms of intoxication, especially in time-long exposure at low doses, metal toxicity is difficult to diagnose. Local skin reactions or systemic symptoms (such as fever, fatigue, and multiple chemical sensitivity) are among the most common clinical signs of a reaction to metals. These symptoms,

however, are not expressed by all exposed individuals, patients often presenting uncommon manifestations, making it harder for the physician to pinpoint a correct diagnosis. Heavy metal exposure has been associated with bone metabolism disorders, atherosclerosis, hypertension and diabetes, and obesity. Studies note that, however, age, gender, and lifestyle may mediate the metabolism of heavy metals. Metal exposure can be confirmed by specific qualitative and quantitative biomarker analysis from target tissues. The analysis of biological fluids such as saliva is noninvasive, fast, and economical, with the mention that metal content tends to appear lower in concentration when time after exposition increases. When choosing proper therapy in treating diseases caused by metals and metal-nanoparticles, biomarker analysis is of utmost importance (Lachowicz et al. 2021).

- Endocrine disruptive chemicals are noted to be involved in the interference with hormone signaling, leading to disease related to the endocrine system and to key metabolic pathways such as the hypothalamus, liver, or pancreas. It is postulated that environmental chemicals promote metabolic changes leading to metabolic disorders. In the literature, there has been an increase in studies associating diabetes and cardiovascular disease with exposure to persistent organic pollutants (Heindel et al. 2017).
- Although a wide range of pesticides have been classified as carcinogenic, pesticides remain the most used substances in agricultural practices. They differ in mode of action and toxicity in the organism. Multiple pesticides can induce genetic damage and adverse effects on the immune, nervous, endocrine, and reproductive systems. Studies have reported through the determination of several cytogenetic biomarkers and oxidative stress parameters, increased DNA damage levels after occupational or environmental pesticides exposure.
- Phthalates are used as plasticizers and additives in numerous products, with human exposure occurring through dietary sources, dermal absorption, and inhalation. Studies have linked phthalate exposure to allergy and asthma, altered reproductive development, sex anomalies, endometriosis, early puberty and fertility, breast and skin cancer, insulin resistance, and type II diabetes. Saliva serves as a biofluid for screening phthalate metabolites (Turesky and Lu 2020).

Evidence suggests that environmental risk for metabolic diseases comes from multifaceted stressors, human populations being exposed to various mixtures of xenobiotics. This is a challenge that has made the toxicologic research keener on single chemical studies rather than the mixed effects of exposure. Moreover, for air pollutants, although there is a growing evidence base for the dose-response relationship, further research is needed due to various influencing factors (Chandra et al. 2022). The simultaneous identification of multiple endogenous biomarkers of effect that is correlated to pathogenic states is important in assessing the overall toxicity risk, providing the most accurate estimation of a subject's true exposure.

When it comes to understanding environmental toxic factors and their impact on human exposure and age-related outcomes, a multidisciplinary collaboration across toxicology, comparative biology, and epidemiology is needed (Malecki et al. 2022).

Conclusions

There are several areas in which salivary analysis of various compounds, such as biomarkers or toxicity markers, has been proven to be practically relevant. Various pathological states such as rheumatic disease, metabolic syndrome, and epilepsy, where the treatment is long term and needs constant monitoring and updating allowed saliva to become an invaluable surrogate matrix preferred in some cases over plasma monitoring.

The pharmacologically active component in serum is generally reflected in the saliva, with the standard analytical methods easily being adapted to accept saliva specimens.

Due to the invasiveness nature of venous blood sampling, the potential for using other biological matrices for the determination of drugs' bioequivalence is thoroughly investigated. There is also a more restrictive clinical setting required for venous blood sampling, a limited number of samples that can be collected, confinement to a laboratory setting, as well as possible clinical and ethical issues that may appear in pediatric or geriatric patients. Therefore, saliva drug monitoring can be an aid in those patients whose clinical status is difficult to assess. Saliva sampling is simple, noninvasive, and can be done repeatedly without any issues, requiring no medical personnel to perform it. Therefore, it is preferred by most patients, and it can be carried out by anyone, including the patients themselves. More so, the ease of sampling oral fluid may represent the basis of a cost-effective approach in screening large populations.

Workplace or roadside saliva sample collection and testing can reduce the legal burdens in obtaining biological samples. The rapid on-site drug screening results, followed by a detailed report of the police officer can be grounds for a future, more invasive blood collection. Oral fluid analysis gives recent, roadside information, shortly after the arrest.

Unlike the case of urine sampling, the medical/legal staff can directly observe the patients when they give the oral fluid samples (to make sure the sample does come from the patient), without the procedure being invasive, all while respecting the patient's dignity and no vulnerability to adulteration.

With applicability in the case of opioid consumption, roadside saliva sample collection comes, though, with some considerations. First, the collection of saliva samples can be contaminated by possible substances that could be found in the oral cavity or by substances in direct contact with the mouth (e.g., THC from smoked cannabis). Secondly, drug concentrations in oral fluid samples are not as well documented as those in blood, which can complicate the interpretation process. More so, compared to plasma samples, oral fluid samples might have a shorter time for the possible detection of substances.

A small number of limitations come with saliva sampling, though: collection from certain patient populations might be difficult, such as the critically ill or those experiencing xerostomia. Furthermore, the samples can be contaminated from gingival bleeding and residues from undissolved tablets. Also, the flow rate, consistency, and the volume of oral fluid may vary from patient to patient.

Overall, oral fluid sample analysis is a very promising tool, with significant potential to be used in the future in emergency rooms, schools, in the workplace, and even in drug rehabilitation centers or roadside by law enforcement officers. There is still more research needed as the complexity of biological fluids and the presence of analytes at trace concentrations require the need for fast, efficient, specific, and quantifiable methods for their determination. Newer analyzing methods depend upon their high selectivity, sensitivity, and capability of simultaneously determining and quantifying multiple drugs.

Mini-Dictionary of Terms

- **Biomarker of toxicity.** Any compound or phenomenon that contributes to the determination of the toxicity felt by the body after inoculation of a toxic agent.
- **Toxicity.** A change in the normal state of the body caused by a toxic agent.
- **Analytical methods**. Methods used in the detection and/ or monitoring of the concentration of target analytes in real samples.
- **Toxic environmental agent.** Composed of the environment that affects the proper functioning of the body.
- **Oral fluid.** All the fluids found in the oral cavity at one time.
- **Saliva.** The fluid produced and released in the oral cavity by the salivary glands.

Key Facts of Salivary Biomarkers in Toxicology

Toxicity biomarkers are useful in analytical practice.

Saliva is a biological fluid that allows noninvasive analysis of biomarkers of toxicity.

Saliva collection method must be considered when interpreting the results.

Chemical composition of the saliva would be influenced by stored temperature.

Toxicological classification of biomarkers reveals three classes: biomarkers of exposure, response, and susceptibility.

Spectral methods are the oldest and robust analysis methods used for detecting toxicity biomarkers.

High-performance liquid chromatography with tandem mass is one of the most utilized analytical methods, with great versatility and high sensitivity and selectivity.

Electrochemical salivary sensors are characterized by high sensitivity, specificity, short analysis time, low cost, and use of small amounts of sample.

Pollution, drugs, smoking, drug abuse, and alcohol are sources of toxicity to the body.

The exposome concept can define the impact that environmental exposure has on human health and, also, must establish a clear causality and impact of these toxic factors.

The gut microbiome acts as a health mediator of environmental stressors, toxicant mixtures, and neurobiology.

Summary Points

- Saliva sampling is done by minimally invasive methods that are easier for patients to accept.
- Saliva does not require laborious processing before testing, even it is short- and medium-term stored.
- Using saliva increases patient compliance and reduces the overall cost of the analysis.
- Analytical methods for the selective and sensitive detection of salivary biomarkers have been developed recently.
- Numerous studies with applications of salivary detection in the biomedical field have been reported.
- Finding salivary biomarkers to assess toxicity to the body is an important goal for researchers and practitioners.
- Many toxic agents reach the body from the environment, food, or other sources, and the body's response to their action must be evaluated.
- Biological aging is closely related to environmental toxicity by induction of systemic inflammation and oxidative stress.

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Part V

Biomarkers in Specific Conditions or Scenarios

Doxorubicin Cardiotoxicity: Preclinical and Clinical Circulating Protein Markers

31

Li-Rong Yu and Varsha G. Desai

Contents

Abstract

Cardiotoxicity remains one of the major threats to cancer patients treated with the potent anticancer drug doxorubicin. It has become a significant challenge in the posttreatment management of the growing population of cancer survivors. Among several risk factors, the lifetime cumulative dose of doxorubicin is considered a key determinant in the development of cardiotoxicity that can manifest into life-threatening congestive heart failure years after completion of

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treatment. A lack of reliable clinical measures with high sensitivity and specificity to identify early signs of heart failure in cancer patients has led to enormous efforts in cardio-oncology to discover early circulating biomarkers of doxorubicin cardiotoxicity. This chapter discusses promising preclinical and clinical circulating protein markers associated with oxidative stress, endothelial cellular injury, inflammation, immune response, hypertrophy, and fibrosis in the heart that may have the potential to predict the risk of cardiotoxicity in cancer patients treated with doxorubicin-based chemotherapy.

Keywords

Doxorubicin · Anticancer drug · Cardiotoxicity · Cardiac troponins · Natriuretic peptides · Immune response proteins · Circulating Biomarkers · Dexrazoxane · Doxorubicinol · Oxidative stress · Hypertrophy · Fibrosis

Abbreviations

BNP	Brain natriuretic peptide
CBR3	Carbonyl reductase 3
CCL	C-C motif chemokine ligand
CHF	Congestive heart failure
cTnI	Cardiac troponin I
cTnT	Cardiac troponin T
CXCL	C-X-C motif chemokine ligand
DOX	Doxorubicin
DOXol	Doxorubicinol
DXZ	Dexrazoxane
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IFN-γ	Interferon gamma
IL-2	Interleukin-2
IL-8	Interleukin-8
LVEF	Left ventricular ejection fraction
MIF	Macrophage migration inhibitory factor
NOTCH1	Neurogenic locus notch homolog protein 1
NT-proBNP	N-terminal pro-brain natriuretic peptide
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
ROS	Reactive oxygen species
RYR2	Ryanodine receptor 2
SERCA2	Sarco-/endoplasmic reticulum calcium-ATPase 2
sICAM-1	Soluble intercellular adhesion molecule 1
SLC28A3	Solute carrier family 28 member 3
SR	Sarcoplasmic reticulum
vWF	von Willebrand factor

Introduction

Doxorubicin (DOX) is a broad-spectrum chemotherapeutic drug that belongs to the anthracycline group. It is effectively used to treat breast, ovarian, and prostate cancers, as well as Hodgkin's and non-Hodgkin's lymphomas, various types of sarcoma, and leukemia in both adults and children (Singal and Iliskovic 1998). However, its long-term clinical use is limited because of a serious cardiotoxicity that may lead to life-threatening congestive heart failure (CHF) in some cancer patients. Several risk factors such as the total cumulative dose, age, sex, pre-existing cardiovascular risk factors, and combination therapy with other anti-cancer drugs have been proposed in the development of DOX-induced cardiotoxicity. The standard-of-care guidelines recommend routine monitoring of heart function in cancer patients after DOX therapy. However, echocardiography or multi-gated acquisition (MUGA) nuclear scanning technologies have limitations in detecting early subclinical changes in heart function (Zidan et al. 2015). Additionally, traditional cardiac disease markers, troponins, and the N-terminal pro-brain natriuretic peptide (NT-proBNP) lack sensitivity and/or specificity in predicting the risk of cardiotoxicity later in life of cancer patients treated with DOX (Armenian et al. 2014; van Boxtel et al. 2015). Over the past decade, preclinical and clinical studies explored numerous circulating proteins related to the diverse pathophysiologies of cardiovascular diseases to reveal promising predictive markers of cardiotoxicity in cancer patients treated with anthracyclines (Arslan et al. 2013; Desai et al. 2019; Mörth et al. 2021; Putt et al. 2015; Schnackenberg et al. 2016; Todorova et al. 2020; van Boxtel et al. 2015; Yarana et al. 2018; Yu et al. 2018). Such biomarkers may allow optimization of treatment strategies or earlier implementation of cardioprotective measures to alleviate the severity of heart damage during treatment or later in cancer patients.

Cardiotoxicity as a Serious Adverse Effect of Doxorubicin

Doxorubicin-induced cardiotoxicity is mainly categorized into acute and chronic with chronic being further categorized as early-onset or late-onset. Acute cardiotoxicity is a rare event and occurs early during treatment or shortly after drug administration. Acute effects include transient arrhythmia, pericarditis-myocarditis syndrome, hypotension, and electrocardiographic alterations and are generally diminished upon discontinuation of therapy (Carvalho et al. 2009). On the other hand, chronic cardiotoxicity is a dose-dependent progressive decline in cardiac function that can manifest into CHF within a year after completion of treatment (early-onset chronic cardiotoxicity) or years after completion of treatment (late-onset chronic cardiotoxicity) (Mulrooney et al. 2009; Von Hoff et al. 1979).

Proposed Mechanisms of Cardiotoxicity

Doxorubicin-induced chronic cardiotoxicity is a progressive deterioration of heart function that can culminate in irreversible cardiomyopathy. It is a multifactorial phenomenon that involves complex interactions between diverse signaling pathways at the molecular level (Fig. 1). The most prevailing hypothesis of DOX-induced cardiotoxicity is increased oxidative stress due to excessive production of reactive oxygen species (ROS). An elevated level of cardiac ROS has been attributed to one-electron reduction of DOX to a semiquinone radical in reactions catalyzed by mitochondrial NADH dehydrogenase, NADPH-dependent cytochrome P450 reductase, and xanthine oxidase (Doroshow 1983). These highly unstable semiquinone radicals regenerate a parent compound by reducing molecular oxygen to superoxide anion and hydrogen peroxide. Additionally, a strong affinity of DOX for iron with the formation of DOX-iron complex significantly contributes to ROS generation in the presence of oxygen. A greater vulnerability of cardiomyocytes to these potent oxidants is partly due to a poor antioxidant defense and inactivation of selenium-dependent glutathione peroxidase-1 by DOX (Doroshow et al. 1980). Failure of cardiac bioenergetics is one of the major determinants of DOX-induced cardiomyopathy (Tokarska-Schlattner et al. 2006; Wallace et al. 2020). Mitochondrial function is crucial to meet the high energy demand of heart muscle for efficient contractility. However, these subcellular organelles are also the prime target of DOX. The highest abundance of these subcellular organelles (40–50%) in cardiomyocytes compared to other tissues such as liver and kidney (Else and Hulbert 1985; Marin-Garcia et al. 2001) and a low antioxidant defense render the heart more vulnerable to DOX toxicity compared to other organs. In addition to disrupting the electron transport activity of respiratory chain complexes by the binding of DOX with cardiolipin in the inner mitochondrial membrane (Goormaghtigh et al. 1990; Jung and Reszka 2001), a major toxic metabolite of DOX, doxorubicinol (DOXol), can disturb mitochondrial energy metabolism by inhibiting the F_0–F_1 proton pump of the complex V (Olson et al. 1988). The significance of topoisomerase IIβ (Top2β), which is predominantly expressed in adult cardiomyocytes, has also been highlighted in DOX-induced cardiotoxicity by Zhang et al. (2012). These investigators demonstrated an important role of Top2β in initiating a series of molecular events involving DNA damage response, apoptosis, and downstream harmful effects on mitochondrial morphology, oxidative phosphorylation, and biogenesis in the hearts of DOX-treated mice (Zhang et al. 2012). Long-term adverse effects of DOX and DOXol have also been reported in rodent models that showed down-regulation of key Ca^{2+}-regulating proteins (RYR2 and SERCA2) localized to the sarcoplasmic reticulum within cardiomyocytes. These alterations may have implications for impaired Ca^{2+} homeostasis and irregularities in contractility of the heart muscle (Farhad et al. 2016; Ruggeri et al. 2018; Olson et al. 2005; Olson et al. 1988). Collectively, these toxic effects can potentially lead to impaired cardiac function that may progress to late-onset irreversible cardiomyopathy even in the absence of DOX.

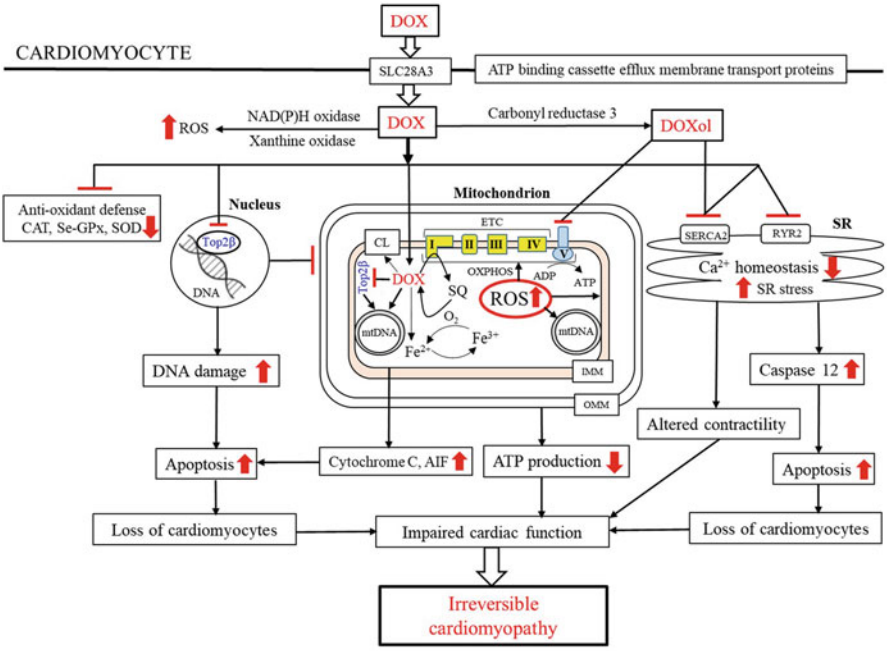

Fig. 1 Schematic illustration of proposed mechanisms of doxorubicin-induced cardiotoxicity. In cardiomyocytes, DOX-induced excessive production of ROS can damage DNA, proteins, and membrane lipids. Oxidative injury to the nDNA and inhibition of Top2β activity by DOX can cause strand breaks in the nDNA, leading to apoptosis (programmed cell death). Within mitochondria, altered Top2β activity can produce strand breaks in mtDNA that encodes key proteins of OXPHOS, thus disrupting energy production. Depletion in mitochondrial energy can also be attributed to inhibition of complex V of the OXPHOS by DOXol. The resulting increase in mitochondrial oxidative stress can induce permeability transition, releasing cytochrome c and AIF to initiate apoptotic process. Doxorubicin and DOXol can also alter Ca^{2+} homeostasis that can consequently lead to cardiac dysfunction. Overall, DOX cardiotoxicity is a multifactorial phenomenon that involves increased oxidative stress, depletion of energy, Ca^{2+} dysregulation, cell death, and loss of cardiomyocytes. These molecular events can steadily deteriorate heart function, eventually progressing to irreversible cardiomyopathy. Additionally, polymorphisms of genes encoding proteins associated with drug influx membrane transport (SLC28A3), ATP-binding cassette efflux membrane transport, drug metabolism (carbonyl reductase 3), and NAD(P)H oxidase multi-enzyme complex may play a major role in DOX pharmacokinetics that can significantly influence the risk of cardiotoxicity. Up arrow indicates increase, down arrow indicates decrease, and ⊣ indicates inhibition. *ADP* adenosine diphosphate; *AIF* apoptosis-inducing factor; *ATP* adenosine triphosphate; *Ca^{2+}* calcium ion; *CAT* catalase; *CL* cardiolipin; *DOX* doxorubicin; *DOXol* doxorubicinol; *ETC* electron transport chain; *Fe^{2+}* ferrous iron; *Fe^{3+}* ferric iron; *IMM* inner mitochondrial membrane; *I, II, III, IV, and V* five multi-subunit complexes of OXPHOS; *mtDNA* mitochondrial DNA; *nDNA* nuclear DNA; *OMM* outer mitochondrial membrane; *OXPHOS* oxidative phosphorylation; *ROS* reactive oxygen species; *RYR2* ryanodine receptor 2; *SERCA2* sarco-/endoplasmic reticulum Ca^{2+}-ATPase 2; *Se-GPx* selenium-dependent glutathione peroxidase; *SLC28A3* solute carrier family 28 member 3; *SOD* superoxide dismutase; *SQ* semiquinone; *SR* sarcoplasmic reticulum; *Top2β* topoisomerase 2β

Potential Risk Factors Associated with Cardiotoxicity

Several risk factors have been proposed in DOX cardiotoxicity (Table 1). Among these, the total cumulative dose of DOX is a major risk factor associated with the development of CHF, with increased prevalence of CHF at total cumulative doses exceeding 500 mg/m^2 body surface area (Swain et al. 2003; Von Hoff et al. 1979). In a retrospective study of 3941 patients treated with DOX, von Hoff et al. (1979) reported CHF in 88 patients (2.2%) within 1 year after completion of treatment. The prevalence of CHF in this cohort was 3% at 400 mg/m^2 that increased to 7% and 18% at 550 mg/m^2 and 700 mg/m^2 total cumulative DOX doses, respectively (Fig. 2). A similar positive correlation between the total cumulative DOX dose and cardiomyopathy was observed in 630 patients with breast or small-cell lung carcinoma (Swain et al. 2003). An estimated percentage of patients with CHF in this cohort was 5%, 16%, and 26% at 400 mg/m^2, 500 mg/m^2, and 550 mg/m^2, respectively, which exponentially increased to 48% at 700 mg/m^2 total cumulative dose. However, signs of cardiotoxic events were also seen in some patients after exposure to $\leq$300 mg/m^2 total cumulative DOX doses in this cohort.

Long-term cardiotoxic effects at total doses $<$400 mg/m^2 have been observed in several other studies in cancer patients treated with DOX. In a study of adult lymphoma patients, a median 300 mg/m^2 cumulative DOX dose resulted in a significant depression in the left ventricular fractional shortening (below 25%) without clinical signs of CHF in 39 of 141 (28%) patients at least 5 years after therapy (Hequet et al. 2004). Furthermore, cardiac susceptibility to total cumulative doses $<$300 mg/m^2 was demonstrated in a large cohort of more than 14,000 adult survivors of childhood or adolescent cancer that received anthracycline-based treatment regimens (Mulrooney et al. 2009). In this cohort, the relative risk of CHF was 2.4-fold higher in patients that received $<$250 mg/m^2 total cumulative doses in comparison with individuals who did not receive anthracyclines. In patients that received $\geq$250 mg/m^2 total cumulative anthracycline doses, the relative risk increased to a

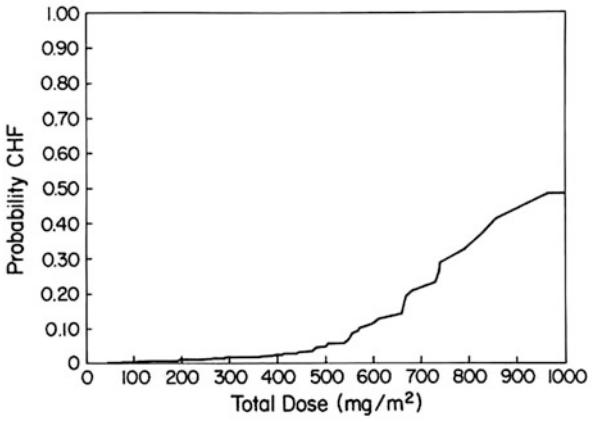

Fig. 2 Cumulative dose-dependent cardiotoxicity. Cumulative probability of developing congestive heart failure (CHF) versus total cumulative dose in mg/m^2 in cancer patients treated with doxorubicin. (Reproduced from Von Hoff et al. (1979) with permission from Annals of Internal Medicine)

Table 1 Potential risk factors associated with anthracycline-induced cardiotoxicity

References	Cohort	Observations	Potential risk factors
von Hoff et al. (1979)	3941 cancer patients who had received doxorubicin ranged from 13 to 5047 mg/m^2 total cumulative dose and were assessed for cardiotoxic events at intervals ranging from 0 to 231 days after the last drug administration	A greater incidence of CHF at higher total cumulative doses	Total cumulative dose, schedule of drug administration
		Lower incidence of CHF with the weekly administration of drug than the single-dose administration every 3 weeks	
Lipshultz et al. (1995)	120 children and adults who had received 244–550 mg/m^2 cumulative doxorubicin doses in childhood at an average of 8.1 years earlier	Higher total cumulative dose associated with increased afterload and decreased left ventricular fractional shortening	Total cumulative dose, female sex, and younger age
		Female patients more vulnerable (greater reduction in contractility) than male patients; a greater sex-based difference at higher cumulative doses	
		Younger age at diagnosis (reduction in left ventricular posterior wall thickness and mass and increased afterload)	
Krischer et al. (1997)	6493 children with cancer who received anthracycline chemotherapy and incidence of clinical cardiotoxicity at a median of 1.3–1.4 years after diagnosis	Total cumulative dose $\geq$500 mg/m^2 increased the risk for cardiotoxicity	Total cumulative dose, female sex, concomitant anthracycline treatment with other anticancer drugs, black race, and trisomy 21
		Female patients at increased risk of early clinical cardiotoxicity than male patients (depressed contractility)	
		A greater risk for cardiotoxicity with combination therapy (mitoxantrone or amsacrine) than the risk associated with anthracycline alone	
		Higher risk for early clinical cardiotoxicity associated with black race	
		Higher risk for early clinical cardiotoxicity among children with trisomy 21	

(continued)

Table 1 (continued)

References	Cohort	Observations	Potential risk factors
Swain et al. (2003)	630 patients with breast or small-cell lung carcinoma treated with doxorubicin	Cumulative dose-related increase in the risk for CHF, with a greater risk at cumulative doses $\geq$500 mg/m^2	Total cumulative dose, older age
		Older patients (age $\geq$65 years) at a greater risk for CHF at total cumulative doses >400 mg/m^2	
Hasan et al. (2004)	100 African American patients treated with doxorubicin-based combination chemotherapy (264–580 mg/m^2 total cumulative dose) and cardiac functional assessment at a median of 1.3 years post-chemotherapy	Three times higher risk for cardiotoxicity in African American patients than the previously reported study population	African American
Hequet et al., 2004	141 patients who had received doxorubicin (250–550 mg/m^2 total cumulative dose) and cardiac functional assessment at least 5 years after chemotherapy	A significant association of male sex, older age, higher total cumulative dose or radiotherapy with development of subclinical cardiomyopathy (fractional shortening below 25%)	Male sex, older age, higher cumulative dose of doxorubicin, and radiotherapy
Blanco et al. (2008)	A nested case-control pilot study in 30 patients with anthracycline-induced CHF (<100 to >500 mg/m^2 total cumulative dose) enrolled in a large cohort of childhood cancer survivors	A 2.6-fold higher formation of DOXol by *CBR3* V244 (G allele) compared with *CBR3* M244 (A allele)	*CBR3* V244M polymorphism
		An 8.0-fold greater risk of CHF among individuals with *CBR3* GG genotype compared with individuals with *CBR3* AA genotype	
Mulrooney et al. (2009)	14,358 5-year survivors of cancer diagnosed under the age of 21 with different types of cancer who had received anthracycline-based chemotherapy	Increased relative risk of CHF at $\geq$250 mg/m^2 total cumulative dose compared with survivors who had not received anthracyclines	Total cumulative dose, female sex, younger age, radiotherapy to chest, length of post-anthracycline follow-up
		Female cancer survivors at a higher relative risk of CHF than male survivors	

(continued)

Table 1 (continued)

References	Cohort	Observations	Potential risk factors
		Higher risk of CHF in survivors diagnosed at age less than 10 years compared with the sibling control group	
		Increased relative risk of CHF in survivors who had cardiac radiation exposure of ≥1500 centigray compared to non-irradiated survivors	
		Increased risk of the cumulative incidence of serious cardiac events in survivors up to 30 years after diagnosis	
Szmit et al. (2014)	208 adult patients with non-Hodgkin's lymphoma treated with doxorubicin-based chemotherapy	Patients with pre-existing hypertension developed left ventricular systolic dysfunction. Other co-existing risk factors in these patients included older age, overweight, hypercholesterolemia, and diabetes	Pre-existing arterial hypertension, older age, overweight, hypercholesterolemia, and diabetes
Hamirani et al. (2016)	549 cancer patients treated with anthracycline and/or trastuzumab	Higher incidence of decline in the left ventricular ejection fraction in patients treated with anthracycline and trastuzumab compared to patients who received anthracyclines alone	Concomitant anthracycline therapy with trastuzumab
Canale et al. (2019)	610 cancer patients treated with anthracyclines and/or trastuzumab: 422 with and 188 without baseline cardiovascular risk factors	Higher incidence of cardiotoxicity in patients with pre-existing cardiovascular disease risk factors compared to patients without cardiovascular disease risk factors	Age, family history of early cardiovascular disease, arterial hypertension, diabetes mellitus, obesity, hypercholesterolemia, and smoking
		Incidence of early-onset cardiac events (< 5 years from the initiation of therapy) with pre-existing cardiovascular risk	

(continued)

Table 1 (continued)

References	Cohort	Observations	Potential risk factors
		factors, whereas late-onset cardiac events (>5 years from the initiation of therapy) without cardiovascular risk factors at baseline	
Vaitiekus et al. (2020)	73 women with breast cancer treated with doxorubicin-based chemotherapy	Pre-existing hypertension significantly correlated with left ventricular systolic dysfunction after completion of therapy	Pre-existing arterial hypertension

Anthracyclines are a class of potent chemotherapeutic drugs that include doxorubicin, daunorubicin, epirubicin, and idarubicin. CHF congestive heart failure; *DOXol* doxorubicinol (a major toxic metabolite of DOX)

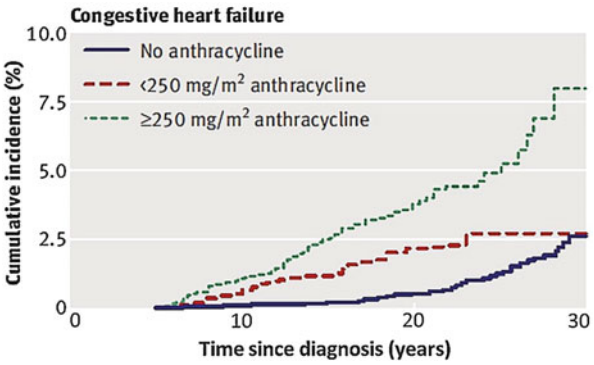

Fig. 3 Cumulative incidence of congestive heart failure by dose and years after diagnosis. A retrospective study of more than 14,000 childhood cancer survivors treated with anthracyclines and monitored up to 30 years after initial diagnosis. (Reproduced from Mulrooney et al. (2009) with permission from the BMJ Publishing Group Ltd)

5.2-fold and showed a relatively greater incidence of CHF at 30 years after cancer diagnosis (Fig. 3). Cardiotoxic potential of a total cumulative DOX dose as low as 45 mg/m^2 was observed in 115 long-term childhood cancer survivors, who showed a significant reduction in the left ventricular mass and dimension at median 11.8 years after the completion of therapy (Lipshultz et al. 2005). These clinical findings clearly suggest that DOX at any dose can manifest into cardiac damage in cancer patients even after completion of the therapy. Identification of high-risk cancer patients for cardiomyopathy will be crucial to initiate appropriate cardioprotective measures to minimize the risk of cardiovascular complications during therapy or later in life.

Technical Barriers for Predicting Early Signs of Cardiac Dysfunction Induced by Doxorubicin

As stated earlier, commonly used clinical techniques such as echocardiography and MUGA scan for evaluation of heart function are less sensitive in detecting early subclinical signs of heart failure in cancer patients after DOX-based chemotherapy. Currently, the potential utility of myocardial strain imaging and cardiac magnetic resonance is being investigated in cancer patients treated with anthracyclines for detecting early structural and functional alterations in the heart that may progress to irreversible damage (Oikonomou et al. 2019; Thavendiranathan et al. 2014). However, these technologies have limitations concerning reproducibility, inter-vendor variability, and/or different software and lack absolute reference standard data (McGregor et al. 2021). In the case of traditional cardiac disease markers, troponins are sensitive markers of myocardial injury and can detect cardiac injury only early during treatment (Ky et al. 2014), whereas there are a few conflicting reports about the use of NT-proBNP in assessing the risk of anthracycline-induced cardiotoxicity in cancer patients during treatment or long-term follow-up (van Boxtel et al. 2015). This highlights a need for identification of novel circulating biomarkers for risk stratification of anthracycline-induced cardiotoxicity to help improve the quality of life of cancer patients. Both preclinical and clinical studies utilized innovative approaches to discover new protein markers that may prove valuable in assessing early cardiac events before irreversible myocardial damage occurs. The following section discusses promising circulating protein markers of DOX cardiotoxicity in preclinical and clinical settings (Table 2).

Circulating Protein Markers of Doxorubicin-Induced Cardiotoxicity

Preclinical Markers of Cardiotoxicity

To date, only a few preclinical studies evaluated circulating protein markers of DOX cardiotoxicity, which are discussed below.

Acute DOX effects, although rare and frequently reversible, can pose a greater risk of cardiotoxicity at high total cumulative doses in some cancer patients (Volkova and Russell 2011). In adult C57BL/6 J male mice, the acute effects of DOX were examined by profiling proteins in serum extracellular vesicles (EVs) after a single 20 mg/kg cardiotoxic dose (Yarana et al. 2018). These authors observed a significant elevation in the level of EVs highly enriched with tissue-specific glycogen phosphorylase (GP) isoforms: brain/heart (PYGB), skeletal muscle (PYGM), and liver (PYGL) in DOX-treated mice. They suggested that the release of these EVs in the

Table 2 Promising circulating protein markers of anthracycline-induced cardiotoxicity

References	Study design/cohort	Type of anthracycline	Time points of marker measurement	Proteins investigated	Predictive markers of cardiac dysfunction
Preclinical studies					
Yarana et al. (2018)	Adult C57BL/6 J male mice (acute cardiotoxicity model – a single 20 mg/kg dose)	Doxorubicin	Protein profiling in serum extracellular vesicles at 1, 24, 48, and 72 h after dose	Proteome by LC-MS/MS	PYGB
Desai et al. (2019)	Adult B6C3F_1 male mice (chronic cardiotoxicity model – 6, 9, 12, 18, and 24 mg/kg total cumulative doses)	Doxorubicin	Protein profiling in plasma at 1 week after each total cumulative dose	1129 proteins using SOMAscan™ proteomic assay	NOTCH1, vWF
Clinical studies					
Sawaya et al. (2012)	81 breast cancer patients	Doxorubicin or epirubicin	At baseline and every 3 months post- anthracycline until 15 months	usTnI, NT-proBNP, ST2	usTnI
Geisberg et al. (2013)	78 breast cancer patients	Not specified	At baseline, after completion of anthracycline therapy or 3 months into trastuzumab therapy	NRG	NRG
Armenian et al. (2014)	150 childhood cancer survivors	Doxorubicin, daunorubicin, epirubicin, idarubicin, mitoxantrone	Two or more years since completion of chemotherapy	BNP, NT-proBNP, troponin T, ST2, gal-3	NT-proBNP
Ky et al. (2014)	78 breast cancer patients	Doxorubicin	At baseline, 3 months, and 6 months	usTnI, hs-CRP, NT-proBNP, GDF-15, MPO, PlGF, sFlt-1, gal-3	usTnI, MPO

Putt et al. (2015)	78 breast cancer patients	Doxorubicin	At baseline and every 3 months for up to 15 months	hs-cTnI, hs-CRP, NT-proBNP, GDF-15, MPO, PlGF, sFlt-1, gal-3	MPO, GDF-15, PlGF
Di et al. (2018)	129 leukemia patients	Doxorubicin, daunorubicin, epirubicin, idarubicin, mitoxantrone, pirarubicin	A day before and the day after chemotherapy	GPBB, Mb, BNP	GPBB, Mb, and BNP in combination
Frères et al. (2018)	45 breast cancer patients	Epirubicin	At baseline, after 2 cycles, at the end of the chemotherapy, 8 days before surgery, 3 months after the surgery	cTnT, NT-proBNP, sST2	sST2
Yu et al. (2018)	27 breast cancer patients	Doxorubicin	At baseline and after the first and the second cycles of chemotherapy	40 chemokines, 9 MMPs, and 33 cardiovascular disease biomarkers including BNP, NT-proBNP, cTnI, GDF-15, Mb, MPO, CRP, vWF	CCL23
Todorova et al. (2020)	51 breast cancer patients	Doxorubicin	At baseline and 2 weeks after the first cycle of chemotherapy	CRP, MPO, TM, TAT complex, P-selectin, vWF	MPO, TM, TAT complex
Mörth et al. (2021)	95 diffuse large B-cell lymphoma patients	Doxorubicin	At diagnosis, during treatment, and after completion of therapy	182 proteins from OLINK® panels, NT-proBNP, troponin I, CRP	SPON-1, IL-1RT1

BNP brain natriuretic peptide; *cTnT* cardiac troponin T; *hs-CRP* high-sensitivity c-reactive protein; *hs-cTnI* high-sensitivity cardiac troponin I; *Gal-3* galectin-3; *GDF-15* growth differentiation factor-15; *GPBB* glycogen phosphorylase, brain isoform; *IL-1RT1* interleukin-1 receptor type 1; *LC-MS/MS* liquid chromatography/tandem mass spectrometry; *Mb* myoglobin; *MMP* matrix metalloproteinase; *MPO* myeloperoxidase; *NRG* neuregulin; *NOTCH1* neurogenic locus notch homolog protein 1; *NT-proBNP* N-terminal pro-brain natriuretic peptide; *PlGF* placental growth factor; *PYGB* glycogen phosphorylase, brain/heart isoform; *sFlt-1* soluble fms-like tyrosine kinase receptor-1; *SPON-1* spondin-1; *sST2* soluble ST2 (interleukin-1 receptor-like 1); *TAT* thrombin-antithrombin complex; *TM* thrombomodulin; *usTnI* ultrasensitive troponin I; *vWF* von Willebrand factor

circulation might be from the respective tissues as these tissues were depleted in their GP isoforms. Among these, the PYGB was proposed as an early marker of cardiac injury as the level of EVs enriched with PYGB was significantly higher at 24 h posttreatment prior to increased levels of cardiac troponin I (myocardial injury marker) in the serum of DOX-treated mice at 72 h posttreatment compared to saline-treated controls (Yarana et al. 2018).

Neurogenic Locus Notch Homolog Protein 1 and von Willebrand Factor

Doxorubicin-induced pathological manifestations in the heart after completion of treatment are a serious concern in cancer survivors. To address the lack of predictive markers of cardiac damage, Desai et al. (2019) profiled 1129 proteins in the plasma of adult $B6C3F_1$ male mice chronically treated with clinically relevant DOX doses (6, 9, 12, 18, and 24 mg/kg total cumulative doses). They revealed six proteins were significantly elevated in plasma before the onset of myocardial injury at 12 mg/kg and higher total cumulative DOX doses. However, only the plasma levels of NOTCH1 (involved in cardiac repair) and vWF (endothelial cell injury marker) were significantly attenuated in DOX-treated mice that also received a cardioprotective drug, dexrazoxane (Fig. 4). Interestingly, a significant decline in plasma NOTCH1 and vWF levels by dexrazoxane concurred with mitigation of DOX-induced lesions only in the heart, whereas pathological manifestations of DOX in the testis, spleen, and bone marrow remained unchanged. This suggests that the release of NOTCH1 and vWF in circulation after DOX exposure may be of cardiac origin, and thus these proteins were proposed as candidate early circulating biomarkers of DOX cardiotoxicity (Desai et al. 2019).

Additional preclinical efforts in collaborative cross mouse strains (Zeiss et al. 2019) may provide better insights into the implications of genetic diversity in differential vulnerability of the heart to toxic DOX effects as seen in cancer patients. Such novel approaches may also facilitate the discovery of new early circulating preclinical markers of cardiotoxicity that may have potential use in clinics to identify high-risk individuals for cardiac damage after DOX treatment.

Clinical Markers of Cardiotoxicity

Traditional Cardiac Disease Markers

Cardiac troponins: Cardiac troponins have been investigated for over two decades as biomarkers of clinical cardiotoxicity. Both cardiac troponin I (cTnI) and cardiac troponin T (cTnT) are of great interest since they are protein components of the sarcomere, the contractile unit within cardiomyocytes. Early studies found that elevated levels of cTnI in the plasma within 72 h after a high-dose chemotherapy administration significantly correlated with a decrease of the left ventricular ejection fraction (LVEF) within 7 or 12 months, and patients with positive cTnI were more likely to experience heart failure (Cardinale et al. 2000; Cardinale et al. 2002). Positive serum levels of cTnT defined as $\geq$ 0.03 ng/mL (measured using Elecsys Troponin T Stat, Roche Diagnostics, Indianapolis, IN, USA) were also found to be

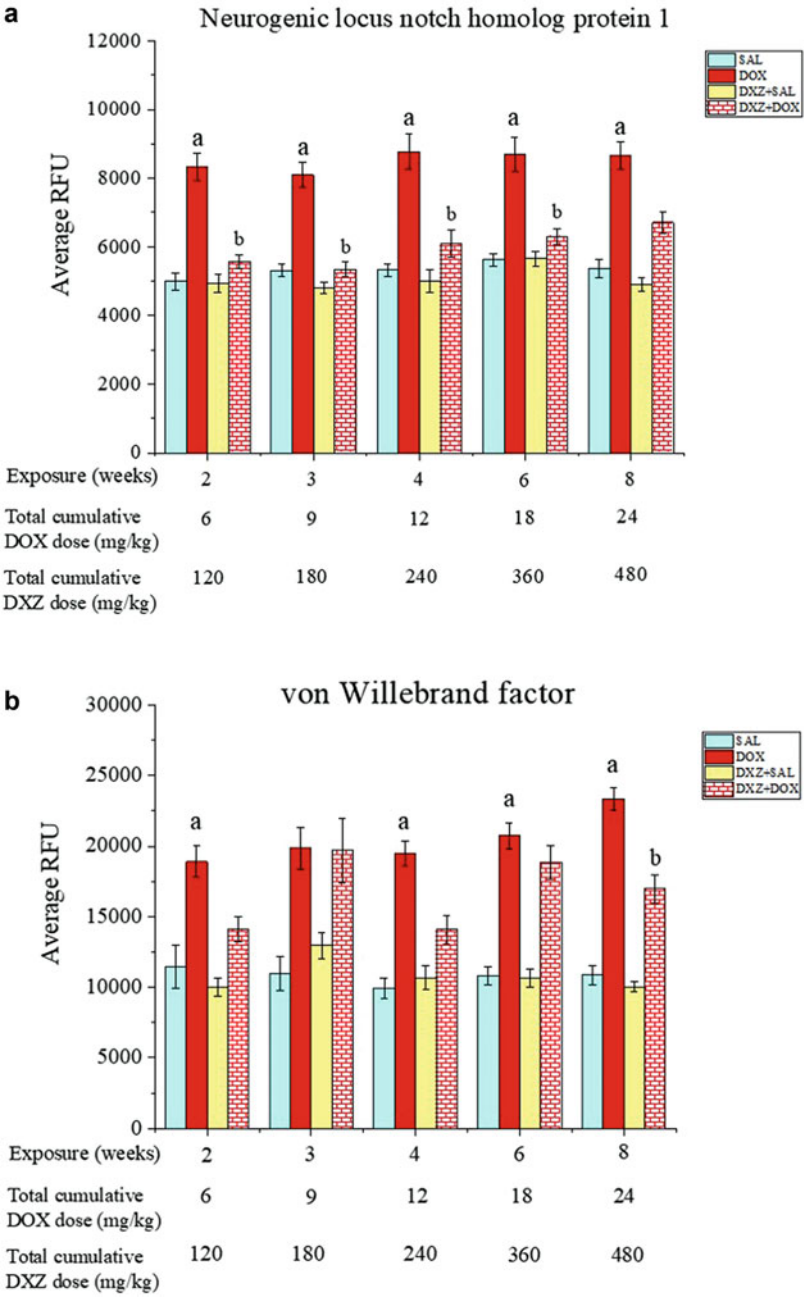

Fig. 4 Effect of doxorubicin on plasma levels of NOTCH1 and vWF and mitigation by dexrazoxane in mice. Average relative fluorescence units measured for NOTCH1 (4a) and vWF (4b) in plasma of male B6C3F$_1$ mice at 1 week after receiving DOX, SAL, DXZ + SAL, or DXZ + DOX. DOX- or SAL-treated mice received a weekly intravenous dose of 3 mg/kg DOX

significantly associated with a greater decrease in LVEF at 6 months after treatment (Auner et al. 2003). Although these studies established the relationship of serum or plasma levels of cardiac troponins with decreased cardiac function within a short period of time (within 12 months), i.e., early onset of cardiotoxicity, the predictive capability of these biomarkers for late-onset cardiotoxicity has not been established. In a study to investigate chemotherapy-induced cardiac dysfunction at a 3-year follow-up, a greater incidence of cardiac events was observed in patients with persistent elevation of cTnI up to 1 month after the last administration of high-dose chemotherapy (Cardinale et al. 2004). A later study found that ultrasensitive cTnI measured at the completion of anthracycline treatment predicted the subsequent development of cardiotoxicity within 15 months (Sawaya et al. 2012). However, a study in anthracycline-exposed childhood cancer survivors for over 10 years found that cTnT did not correlate with cardiac dysfunction (Armenian et al. 2014). A recent meta-analysis of troponins for the prediction of cardiotoxicity included 17 studies (2163 patients) for dichotomous data and 6 studies (230 patients) for absolute troponin levels (Michel et al. 2020). The meta-analysis found that the incidence of LVEF impairment was higher in patients with elevated troponins compared to troponin-negative patients, especially for patients undergoing high-dose regimens or patients receiving anthracyclines and/or HER2 inhibitor therapy. It also revealed that the association between elevated troponins and the left ventricular (LV) dysfunction was detected more consistently in studies investigating high-dose anthracycline treatment ($\geq$ 240 mg/m^2 doxorubicin equivalent dose) as compared to the low-dose therapy. The overall sensitivity and specificity for the diagnostic value of troponins were 69% and 87%, respectively. In addition, the study also found that high-sensitivity troponin analysis was not superior to conventional troponin analysis for the prediction of LV dysfunction and had no enhanced sensitivity and specificity in detecting decreased LVEF. On the other hand, some studies revealed that there was no correlation of serum/plasma levels of cTnI or cTnT with reduced LVEF (Feola et al. 2011; Gulati et al. 2017). In addition, it was also found that elevation of serum cTnI or cTnT was not detected at 1 or 2 years after chemotherapy (Feola et al. 2011; Armenian et al. 2014). Taken together, these results suggest that further studies are required to evaluate cardiac troponins (e.g., timing of testing) for predicting cardiotoxicity, especially late-onset chronic cardiotoxicity.

Fig. 4 (continued) dose or an equivalent volume of SAL for 2, 3, 4, 6, and 8 consecutive weeks, resulting in total cumulative DOX doses of 6, 9, 12, 18, and 24 mg/kg, respectively. (DXZ + SAL)- or (DXZ + DOX)-treated mice received an intraperitoneal dose of 60 mg/kg DXZ 30 min before administration of SAL or DOX for 2, 3, 4, 6, and 8 consecutive weeks, resulting in total cumulative DXZ doses of 120, 180, 240, 360, and 480 mg/kg, respectively. Data are presented as mean ± standard error of the mean (SEM; N = 5–11 plasma samples/group). [a]FDR < 0.1, statistically significant difference between DOX- and SAL-treated mice (indicated DOX effect). [b]FDR < 0.1, statistically significant difference between (DXZ + DOX)- and DOX-treated mice (indicated DXZ-mediated mitigation of DOX effect). *DOX* doxorubicin; *DXZ* dexrazoxane; *FDR* false discovery rate; *RFU* relative fluorescence unit; *SAL* saline

Brain natriuretic peptide and N-terminal pro-brain natriuretic peptide: Expression of brain natriuretic peptide (BNP) is increased upon cardiomyocyte injury or cardiac overload and mechanical stress. Both BNP and N-terminal proBNP (NT-proBNP) have been examined for diagnosis or prognosis of chemotherapy-induced cardiotoxicity. Studies indicated that an early increase of NT-proBNP (24 h post-drug administration) was observed after patients received DOX-based chemotherapy (Broeyer et al. 2008), and a persistent increase of NT-proBNP during the first 72 h of high-dose chemotherapy was significantly associated with a reduction in LVEF within 12 months (Sandri et al. 2005). In another study of lower-dose chemotherapy, persistently elevated NT-proBNP (24 h after each treatment cycle) was also significantly associated with a decrease in LVEF within 12 months (Romano et al. 2011). Furthermore, elevated BNP (>100 pg/mL), measured when half of the cumulative dose of anthracyclines was administered, was predictive of heart failure with an area under the curve (AUC) of 0.77 (95% CI of 0.65–0.90) (Skovgaard et al. 2014). However, other studies found that elevated serum/plasma levels of BNP and NT-proBNP during chemotherapy were not significantly associated with cardiac dysfunction (Dodos et al. 2008; Gulati et al. 2017). A recent meta-analysis of 10 individual studies (462 patients) revealed that an elevated absolute mean BNP/NT-proBNP levels in patients with LV dysfunction were only present in the anthracycline-treated subgroup and elevated BNP/NT-proBNP was not consistently associated with decreased LVEF (Michel et al. 2020). The observation that increased levels of NT-proBNP returned to baseline after each cycle of anthracycline therapy (Broeyer et al. 2008) suggests that the timing of blood sample collection for testing this biomarker is critical. Although several studies showed the relationship between the levels of natriuretic peptides and late-onset cardiotoxicity (Tan and Lyon 2018), the association of early increases of BNP/NT-proBNP with late-onset cardiotoxicity has yet to be further examined and validated.

Promising Markers of Cardiotoxicity

Many clinical studies, primarily in breast cancer patients, explored circulating molecular markers to address the knowledge gaps concerning reliable early predictive biomarkers of anthracycline-induced cardiotoxicity. Biomarkers related to oxidative stress, inflammation, apoptosis, cardiac hypertrophy, fibrosis, vascular dysfunction, and myocardial remodeling are gaining more attention to examine their potential prognostic value in assessing early events of DOX cardiotoxicity. A few of these promising protein markers of DOX cardiotoxicity are discussed below.

Myeloperoxidase: Myeloperoxidase (MPO) possesses pro-inflammatory and pro-oxidant properties and has been implicated in the pathogenesis of cardiovascular diseases (Patoulias et al. 2021). Bearing in mind the significance of oxidative stress in the development of DOX cardiotoxicity, the potential of MPO in assessing the risk of cardiotoxicity was evaluated in breast cancer patients treated with DOX (0–3 months) followed by trastuzumab (3–15 months) (Putt et al. 2015). These authors correlated plasma MPO concentrations relative to baseline values with echocardiography measures at every 3-month visit over the course of 15 months of therapy. A significant association between increased MPO concentration with an

increased risk of cardiotoxicity at the same visit (hazard ratio 1.37, 95% CI 1.11–1.69; $p = 0.02$) and subsequent visits at every 3 months (hazard ratio 1.32, 95% CI 1.11–1.58; $p = 0.003$) led authors to suggest MPO as a useful biomarker for risk stratification of cardiotoxicity in breast cancer patients treated with DOX-based chemotherapy. Todorova et al. (2020) also reported MPO as a prognostic biomarker of DOX cardiotoxicity as the plasma MPO level was higher at baseline and after the first cycle of chemotherapy in breast cancer patients that showed significantly lower LVEF compared to breast cancer patients with normal heart function after four cycles of chemotherapy (Todorova et al. 2020).

Growth differentiation factor-15: Growth differentiation factor-15 (GDF-15) is highly expressed in myocardium and endothelial cells and has been indicated as a novel biomarker of heart failure and coronary artery disease (Anand et al. 2010; Xu et al. 2011). In addition to MPO discussed above, Putt et al. (2015) also proposed GDF-15 as an early biomarker of DOX cardiotoxicity because of a significant correlation between an increased GDF-15 level in plasma and a higher risk of cardiotoxicity at the same visit (hazard ratio 1.80 (95% CI 1.20–2.69), $p = 0.007$) and at subsequent 3-month visits (hazard ratio 1.59 (95% CI 1.06–2.40), $p = 0.02$) in breast cancer patients treated with DOX-based chemotherapy (Putt et al. 2015). In a study of childhood cancer survivors treated with anthracyclines, the average plasma GDF-15 level was significantly higher after completion of treatment compared to age- and gender-matched healthy volunteers (Arslan et al. 2013). However, there was a lack of a significant difference in the left ventricular systolic function or average plasma cTnI levels between cancer survivors and healthy controls. This led the authors to suggest GDF-15 as a biomarker of severity of cardiotoxicity induced by anthracyclines in childhood cancer survivors.

Galectin-3: Galectin-3 (Gal-3) is significantly involved in pathophysiological conditions of heart disease such as inflammation, fibrosis, tissue repair, and remodeling (Krześlak and Lipińska 2004). Unlike MPO and GDF-15 that showed a significant correlation between their plasma levels and the risk of cardiotoxicity at the same visit and at subsequent 3-month visits, a significant association between plasma Gal-3 level and the risk for cardiotoxicity (hazard ratio 1.60 (95% CI 1.12–2.28), $p = 0.04$) was observed only at subsequent 3-month visits over the 15 months of treatment in breast cancer patients treated with DOX-based chemotherapy (Putt et al. 2015). Contrary to these findings, there was a lack of correlation between plasma Gal-3 level and the left ventricular dysfunction after anthracycline treatment in childhood cancer survivors (Armenian et al. 2014) or in breast cancer patients 1 year after treatment (van Boxtel et al. 2015).

Neuregulin-1: Neuregulin-1 (NRG-1) belongs to epidermal growth factor superfamily and is a paracrine growth factor that is abundantly expressed in cardiac endothelial cells. It plays an important regulatory role in cardiomyocyte differentiation and cardiac adaptation to physiological stress or injury (Odiete et al. 2012). An independent association of elevated serum level of the β-isoform of NRG-1 (NRG-1β) with cardiac disease severity and risk for serious outcomes has been demonstrated in patients with heart failure (Ky et al. 2009). It was further determined that the potential of NRG-1β in risk stratification could be enhanced in combination

with BNP (measure of cardiac pressure overload). The potential of NRG as a cardiotoxicity marker was also examined in breast cancer patients treated with anthracyclines- and/or trastuzumab-based regimens (Geisberg et al. 2013). Higher baseline levels of NRG in plasma in breast cancer patients with a significant decline in the LVEF after completion of anthracycline treatment led these authors to suggest the usefulness of circulating NRG in identifying patients at high risk for adverse cardiac outcomes.

Immune response proteins: Inflammatory immune responses play important roles in cardiovascular disease development and drug-induced cardiotoxicity (Fabiani et al. 2021). Plasma/serum proteins that represent the inflammation status or drug-induced immunological responses could be early biomarkers of chemotherapy-induced cardiotoxicity. In a study of 27 adult breast cancer patients treated with a combination of DOX and cyclophosphamide every 2–3 weeks for 4 cycles, 5 patients presented with >10% decline of LVEF, 5 patients presented with LVEF decline of 5–10%, and 17 patients maintained normal LVEF at the end of chemotherapy (Yu et al. 2018). Multiplex immunoassays revealed that the patients with abnormal decline of LVEF (>10%) had higher plasma levels of CCL23 before (T0) and after the first (T1) and the second (T2) cycles of DOX-based chemotherapy than the patients who maintained normal LVEF (Fig. 5). The study also found lower levels of CXCL6 and sICAM-1 and higher levels of CCL27 at T0; lower levels of CXCL5, CCL26, CXCL6, GM-CSF, CXCL1, IFN-γ, IL-2, IL-8, CXCL11, CXCL9,

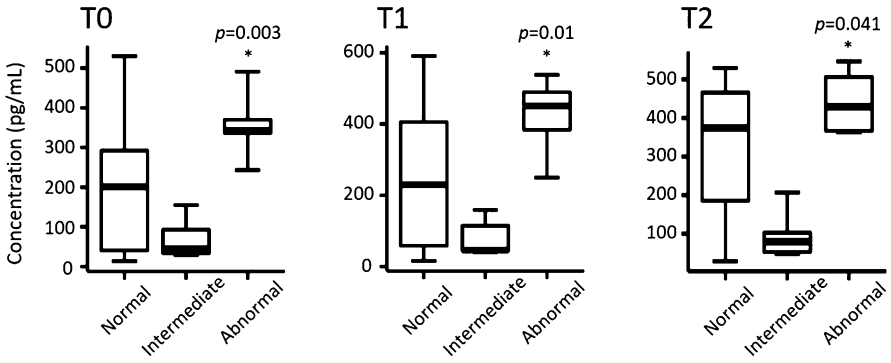

Fig. 5 Differential plasma levels of CCL23 in breast cancer patient groups with normal, intermediate, or abnormal cardiac function after four cycles of DOX-based chemotherapy. Blood samples were collected before (T0) and after the first cycle (T1) and the second cycle (T2) of DOX treatment. An asterisk denotes a statistically significant different level in that group as compared to the normal group. P-values were calculated by Welch's t-test. Edges of boxes denote 25th and 75th percentiles, lines are median concentrations, and error bars are minimum and maximum concentrations. Normal, reduction of left ventricular ejection fraction (LVEF) was <5% after four cycles of DOX treatment; intermediate, asymptomatic reduction of LVEF was 5–10% after four cycles of DOX treatment; abnormal, an asymptomatic reduction of LVEF was >10%, or LVEF was below <50%, or a reduction of LVEF was >5% to LVEF<55% with symptoms of heart failure after completion of four cycles of DOX treatment. (Figure modified from Yu et al. (2018))

CCL17, and CCL25 at T1; and higher levels of MIF at T2 in the patients with decreased LVEF. These immune response proteins were identified at the baseline level or early stage of cancer treatment and, therefore, could be early predictive biomarkers of cardiotoxicity.

Interleukin-1 receptor type 1 and spondin-1: A recent study in 95 diffuse large B-cell lymphoma (DLBCL) patients profiled 182 plasma proteins using OLINK™ multiplex panels (cardiovascular III and oncology II) before, during, and after treatment with DOX-based chemotherapy to identify proteins related to pre-existing or developing cardiovascular disease (Mörth et al. 2021). In this cohort, 32 patients had cardiovascular conditions prior to initiation of treatment, and 22 patients developed cardiovascular disease during follow-up. These authors identified interleukin-1 receptor type 1 (IL-1RT1) and spondin-1 (SPON-1) as novel proteins of interest because of significant differences in their plasma levels between DLBCL patients with and without cardiovascular disease. A significant correlation between elevated IL-1RT1 levels before and during treatment and the risk for cardiovascular disease (fold change 1.24, 95%CI 1.10–1.39, $p = 0.0004$, $q = 0.082$) indicated IL-1RT1 protein as a likely prognostic marker of cardiovascular disease in DLBLC patients treated with DOX. On the other hand, a significant association between higher SPON-1 level in plasma and incidence of cardiovascular disease at diagnosis (fold change 1.22, 95%CI 1.10–1.35, $p = 0.0002$) led authors to propose SPON-1 protein as a potential marker of pre-existing cardiovascular disease in DLBCL patients.

Potential plasma markers of DOX cardiotoxicity discussed above may have functional roles in other organs besides the heart and thus may offer limited cardiac specificity. Further large-scale studies are needed for validation of their predictability of cardiotoxicity in a clinical setting. However, these newly emerging biomarkers representing various cardiac pathophysiologies may act synergistically in multi-biomarker panels that also include traditional cardiac disease markers (highly sensitive troponins and NT-proBNP) to help stratify cancer patients at risk for DOX cardiotoxicity later in life. Continued efforts in cardio-oncology are essential to discover early sensitive and cardiac-specific biomarkers capable of clinically predicting the risk of DOX cardiotoxicity. Such biomarkers will be valuable in early decision-making of suitable chemotherapy regimens or pharmacologic interventions to prevent/limit adverse cardiovascular outcomes in cancer patients treated with DOX. A majority of the studies that explored cardiotoxicity biomarkers primarily focused on adult cancer patients treated with anthracyclines. Additional efforts in biomarker discovery in pediatric oncology patients are of utmost importance to aid in assessing and managing cardiotoxicity in an increasing population of long-term childhood cancer survivors. Integration of early circulating novel cardiotoxicity markers with sophisticated imaging modalities may enhance the prognostic potential in detecting early subclinical dysfunction in cancer patients treated with DOX-based chemotherapy.

Applications to Prognosis

Novel circulating markers that are reviewed in this chapter appear promising in predicting the risk of cardiotoxicity in cancer patients following DOX-based chemotherapy. Glycogen phosphorylase brain/heart isoform, indicated as an early preclinical biomarker of acute DOX cardiotoxicity, showed clinical utility in detecting early signs of acute effects of anthracycline-based chemotherapy before cardiac dysfunction in adult leukemia patients (Di et al. 2018). Likewise, the potential use of vWF, a predictive marker of early-onset DOX-induced chronic cardiotoxicity in mice, in combination with plasma nitrites was shown beneficial in an early assessment of the risk for cardiovascular dysfunction in cancer patients treated with anthracyclines (Giri et al. 2019). Additionally, clinical prognostic markers of cardiotoxicity such as MPO, GDF-15, Gal-3, NRG, IL-1RT1, CCL23, and SPON-1 proposed in adult cancer patients treated with anthracyclines could be evaluated for their potential in pediatric cancer patients, a highly vulnerable population to anthracycline-induced cardiotoxicity. This could lead to the design of multi-biomarker signature panels for assessing the risk for adverse cardiovascular outcomes following anthracycline-based chemotherapy.

Mini-dictionary of Terms

- **Congestive heart failure**: A condition of the heart to inadequately pump blood to meet the body's demand.
- **Doxorubicinol**: A secondary alcohol metabolite of doxorubicin. It is more toxic than doxorubicin itself.
- **Left ventricular ejection fraction**: The percentage of blood volume pumped out of the left ventricle of the heart with each heartbeat. It is the measure of heart function.
- **Mitochondrion**: A cellular organelle responsible for generation of most of the chemical energy in the form of adenosine triphosphate by oxidative phosphorylation for cell functions.
- **Oxidative stress**: A condition within the cell when the by-products of oxygen metabolism exceed the cell's capacity to detoxify them.
- **Proteomics**: Identification, quantitation, or characterization of the total proteins expressed in a cell, tissue, or organism.

Key Facts of Doxorubicin Cardiotoxicity

- Cardiotoxicity is one of the serious side effects of doxorubicin in cancer patients.
- A lifetime total cumulative dose of doxorubicin is a major risk factor involved in the development of cardiomyopathy.

- Acute cardiotoxicity of doxorubicin is rare and reversible but may pose a threat to the heart at higher total cumulative doses.
- Adverse effects of doxorubicin in heart can occur within a year or decades after completion of therapy that may lead to congestive heart failure in some cancer patients.
- Dexrazoxane is a cardioprotective agent that has been effectively used to reduce the severity and the incidence of toxicity in the hearts of cancer patients treated with doxorubicin.

Summary Points

- Long-term clinical use of DOX in cancer patients is limited by a serious cardiotoxicity.
- Cardiovascular complications years after completion of DOX treatment are a major concern in a growing population of cancer survivors.
- Conventional techniques are less sensitive or specific in predicting the risk for cardiac damage later in life in cancer patients treated with doxorubicin-based chemotherapy.
- Cardio-oncology research currently focuses on identification of novel circulating biomarkers for early risk assessment of cardiotoxicity in patients/survivors treated with doxorubicin.
- Preclinical and clinical research revealed several early promising circulating protein markers of cardiotoxicity related to oxidative stress, endothelial cell injury, immune response, inflammation, and fibrosis.
- Integration of new circulating protein markers with advanced imaging tools will improve the risk stratification of cardiotoxicity in cancer patients.

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Biomarkers of Sulfur Mustard (Mustard Gas) in Urine

32

Sermet Sezigen

Contents

Abstract

Sulfur mustard (SM) is a banned chemical warfare agent, and it mainly affects the eyes, the respiratory tract, and the skin as an alkylating vesicant. After a symptom-free latency period which depends on the total absorbed dose, typical ocular, respiratory, cutaneous, and hematological symptoms of SM exposure could be observed. There is no antidote or a specific pharmacological agent available for the treatment of SM poisoning, so the treatment is still symptomatic after decontamination of chemical casualties. The verification of SM exposure in biomedical samples including the urine, blood, and tissues by using instrumental analysis techniques like gas or liquid chromatography with tandem mass spectrometry is essential for diagnostic and forensic purposes. Urinary metabolites including glutathione and DNA products are unambiguous biomarkers of SM poisoning. The chapter discusses the bioanalysis of urinary metabolites of SM by

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addressing the metabolism of SM, chronological development of instrumental analysis techniques, and recent bioanalytical approaches.

Keywords

Chemical warfare agents · Chemical terrorism · Chemical casualty · Sulfur mustard · Bioanalysis · Urinary metabolites · β-Lyase metabolites · DNA adduct · Protein adduct · Gas chromatography · Liquid chromatography

Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CWA	Chemical warfare agent
CWC	Chemical Weapons Convention
DNA	Deoxyribonucleic acid
GC-MS/MS	Gas chromatography in combination with tandem mass spectrometry
GSH	Glutathione
HD	Distilled sulfur mustard
HETE-Val	HETE-valine
IL	Interleukin
LC-MS/MS	Liquid chromatography in combination with tandem mass spectrometry
LOD	Low limit of detection
MAP-1	Monocyte-activating protein-1
MSMTESE	1,1′-Methylsulfinyl-2-[2-(methylthio)-ethylsulfonyl]ethane
N^3-HETEA	N3-[2-[(2-hydroxyethyl)thio]ethyl]-adenine
N^7-BisG	Bis[2-(guanin-7-yl)ethyl]sulfide
N^7-HETEG	N7-[2-[(2-Hydroxyethyl)thio]-ethyl]guanine
NAD^+	Nicotine adenine dinucleotide
NF-κB	Nuclear factor-κB
O^6-HETEG	O6-[2-[(2-Hydroxyethyl)thio]ethyl]-guanine
OPCW	The Organisation for the Prohibition of Chemical Weapons
PARP	Poly(ADP-ribose) polymerase
POC	Point of care
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SBMSE	1,1′-Sulfonylbis[2-(methylsulfinyl)ethane]
SBMTE	1,1′-Sulfonylbis[2-(methylthio)ethane]
SBSNAE	1,1′-Sulfonylbis[2-S-(N-acetylcysteinyl)ethane]
SM	Sulfur mustard
TDG	Thiodiglycol
TDGO	Thiodiglycol sulfoxide
TNFα	Tumor necrosis factor α
WWI	World War I

Introduction

Chemical warfare agents (CWAs) are synthetic toxic chemicals which are still being used for causing death, injury, or other physical and physiological harms through chemical terrorist attacks or targeted assassinations. Munitions and devices, which are specifically designed for weaponing a toxic chemical, are also included into the content of chemical weapons (OPCW 2021a).

The Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and on Their Destruction (CWC) which entered into force on April 29, 1997, lists certain toxic substances including derivatives of chemical warfare agents and their precursors in Schedules 1, 2, and 3 (OPCW 2021b). The Organisation for the Prohibition of Chemical Weapons (OPCW) is the implementing body for the CWC which has been signed and committed by 193 State Parties (OPCW 2021c).

CWAs are generally classified according to their physiological effects on target organs and tissues as nerve agents, vesicants (blistering agents), lung-damaging agents (choking agents), blood agents (cyanides), incapacitants, and riot control agents (Szinicz 2005; Balali-Mood et al. 2016). Vesicants including sulfur mustard (SM), nitrogen mustards, and lewisites are corrosive toxic compounds which mainly affect skin and cause typical lesions of chemical burns. Eyes and airways are also characteristic target organs of vesicants (Szinicz 2005; Ganesan et al. 2010; Schwenk 2018). SM is the most important threat in the vesicant group of CWAs with its highest military significance (Kehe and Szinicz 2005).

SM is a functional alkylating vesicant CWA. It has cytotoxic, teratogenic, mutagenic, and carcinogenic effects on human beings. Besides, it is also known as a radiomimetic agent because some action mechanisms of SM behave like ionizing radiation (Malhotra et al. 1999). While "H" is the code name of SM which is synthesized with "Lewinstein process," "HD" is the code name of distilled mustard (Kehe and Szinicz 2005, Ghaednia Jahromy et al. 2017). Pure SM is a light-yellow oily liquid which is almost odorless where HD is yellow to dark brown. Characteristic odor of technical grade distilled SM smells like garlic, horseradish, or mustard because of additional impurities, so it is called as "mustard gas" (Malhotra et al. 1999; Kehe and Szinicz 2005). Victims of SM exposure reported that they felt garlic or mustard odor during the chemical attacks (Sermet et al. 2019; Sezigen et al. 2020a), and it is mostly resistant to outer conditions. Physicochemical properties of SM are presented in Table 1.

History

SM was first investigated by César Despretz in 1822. Frederick Guthrie and Albert Niemann synthesized SM in different studies in 1860. German chemist Viktor Meyer developed "Meyer method" which was used for large-scale production of pure SM in 1896 by the chlorination of thiodiglycol with phosphorus trichloride (Malhotra et al. 1999; Steinritz and Thiermann 2016). Hans Thacher Clarke and Emil Fischer

Table 1 Physicochemical properties of sulfur mustard (Malhotra et al. 1999; Kehe and Szinicz 2005; Ganesan et al. 2010)

	Sulfur mustard
CAS number	505-60-2
Chemical formula	$C_4H_8C1_2S$
Molecular weight	159.08
Liquid density (g/cm^2 at 25 °C)	1.27
Vapor density (air = 1)	5.4
Freezing point (°C)	14.45
Boiling point (°C)	228
Vapor pressure (mmHg at 20 °C)	0.115
Volatility (mg/m^3 at 25 °C)	610
Viscosity (poise) at 20 °C	0.459

focused on using SM as a CWA before the Great War. They modified the Meyer process and they used hydrochloric acid (HCl) in the reaction with thiodiglycol (Szinicz 2005; Ghabili et al. 2011; Ghaednia Jahromy et al. 2017).

In modern history of weapons of mass destruction, World War I (WWI) was the first military conflict that CWAs were used in large-scale chemical attacks. Respiratory irritants were the first CWAs that were used in August 1914. Following respiratory irritants, lung-damaging agents including chlorine and phosgene were used. Blister agent mustard gas was first used near Ypres, Belgium, on July 12 and 13, 1917, by German forces against British and Canadian forces (Szinicz 2005; Sezigen et al. 2020a). After the attack, allied forces mostly suffered from eye and lung injuries. The large number of allied chemical casualties and severity of SM lesions caused overwhelming of medical resources. CWAs that were especially used in the Western Front killed approximately 100,000 and injured more than 1,200,000 people in WWI (Ganesan et al. 2010). Mustard gas injured approximately 186,000 soldiers in British Army with a death toll of 2.6%, and this number was 80% of all chemical victims of Britain. Mustard gas caused more chemical casualties than other CWAs including lung-damaging agents and cyanides, so it was called as "King of Battle Gases" (Fitzgerald 2008). It was estimated that over 400,000 soldiers had prolonged medical care after WWI due to delayed effects of SM exposure (Balali-Mood and Hefazi 2005).

SM was extremely used against civilians by Spanish Armed Forces during the Rif War between 1921 and 1926 (Balali-Mood and Abdollahi 2015, 32–37). SM was also used during the invasion of Ethiopia by Italian Armed Forces in 1935 and 1936. Imperial Japanese Army used SM in China before and during the World War II. Egypt forces used SM in Yemen between 1963 and 1967. Iraqi Air Force used SM against civilians in a northern Iraq city Sardasht on June 28, 1987. It was estimated that more than 4,500 civilians were exposed to SM during the chemical attacks (Ghabili et al. 2011; Balali-Mood and Abdollahi 2015, 32–37; Ghaednia Jahromy et al. 2017). SM was used in various chemical attacks by Iraqi forces during the Iran-Iraq War. More than 100,000 Iranians were exposed to SM, and 34,000 of them suffered from chronic effects of the exposure (Khateri et al. 2003; Kehe and Szinicz 2005).

During the Syrian Civil War which began in 2011, CWAs were used against civilians by state forces and non-state actors in Syria and Iraq (OPCW 2015, 2018, 2019; Zubel et al. 2018; Sezigen and Kenar 2019, 2020; Sezigen et al. 2020a). The first large-scale chemical attack, which nerve agent sarin was used, killed more than 1,400 civilians early in the morning on August 21, 2013, in Damascus, Syria (Mangerich and Esser 2014). Besides chlorine, non-state actors used SM against civilians in Syria (Kilic et al. 2018, Sezigen et al. 2019).

Toxicokinetics of Sulfur Mustard

SM is absorbed through the eyes, respiratory tract, and skin (Sezigen and Kenar 2020). Kinetics of SM was investigated in various studies which used several kinds of animal models including the rat, hairless guinea pig, small pig, and marmoset monkey. Animals were exposed to SM through intravenous, subcutaneous, and inhalational routes (Read 2016; Worek et al. 2016). Accidental contacts or terrorist attacks were major SM exposures in humans (Barr et al. 2008; Sezigen et al. 2019).

Liquid SM or mustard gas could penetrate from human skin at a rate of 1–4 mg/cm^2/min at 21 °C. Although SM is a lipophilic agent, it was shown that only 20% of applied liquid SM was absorbed through the skin. Penetration rate could change according to outer temperature and humidity. It was estimated that 12% of absorbed SM remained in the skin and 88% was systemically absorbed through the circulation as free SM. While 10–20% of total SM that is penetrated through the skin binds covalently to cellular macromolecules, the remaining SM (80–90%) is transferred into circulatory system (Chilcott et al. 2000; Kehe and Szinicz 2005; Balali-Mood and Hefazi 2005).

There are four metabolic pathways of SM metabolism identified in animal models including rat and mouse (Xu et al. 2014). However, there are not enough data on metabolic pathways of SM in human models. SM as an alkylating agent reacts quickly with water, glutathione, and macromolecules such as proteins and DNA via hydrolase, oxidation, and conjugation.

The first metabolic pathway is hydrolysis and oxidation of SM. SM undergoes internal cyclization in order to form sulfonium group which alkylates many biomolecules. As SM is a bifunctional molecule, both chlorines can be leaving groups (Malhotra et al. 1999; Ghabili et al. 2011; Balali-Mood and Abdollahi 2015). The major urinary metabolites of hydrolytic breakdown are thiodiglycol (TDG) and its oxidative analogue thiodiglycol sulfoxide (TDGO) as shown in Fig. 1 (Boyer et al. 2004; Xu et al. 2014; Golime et al. 2019). Figure 1 also shows that the reaction with oxidizing agents converts SM directly to sulfoxide or sulfone which constitutes detoxification (Malhotra et al. 1999; Ghabili et al. 2011).

The second metabolic pathway consists of glutathione adducts of SM through a series of reactions including glutathione conjugation, oxidation, and β-lyase cleavage as shown in Fig. 2 (Eyison et al. 2019, Golime et al. 2019, Cheng et al. 2021). The glutathione-derived metabolite 1,1′-sulfonylbis[2-S-(N-acetylcysteinyl)ethane] (SBSNAE), β-lyase metabolites 1,1′-sulfonylbis[2-(methylsulfinyl)ethane] (SBMSE),

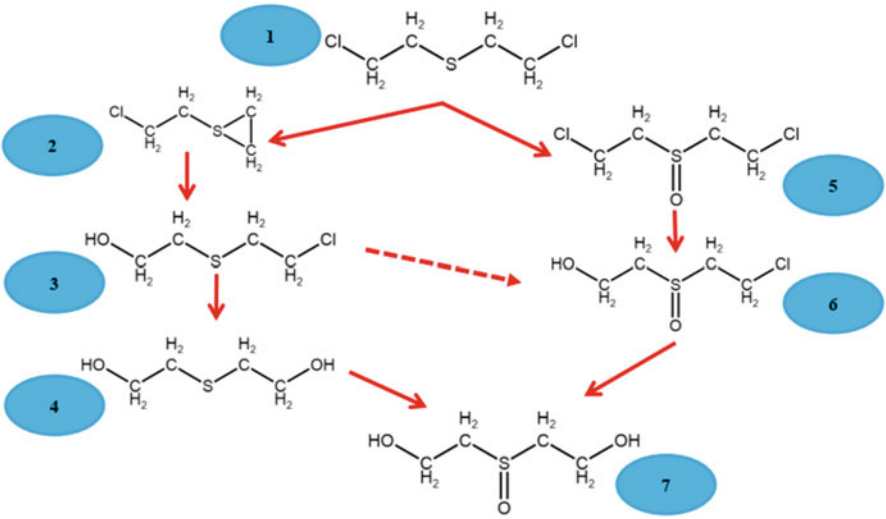

Fig. 1 Hydrolysis/oxidation of SM and major urinary metabolites of the pathway. (1, Sulfur mustard (SM); 2, sulfonium ion; 3, hemimustard; 4, thiodiglycol (TDG); 5, bis-β-chloroethyl sulfoxide (SMO); 6, 2-Chloroethyl 2-hydroxyethyl sulfide; 7, thiodiglycol sulfoxide (TDGO))

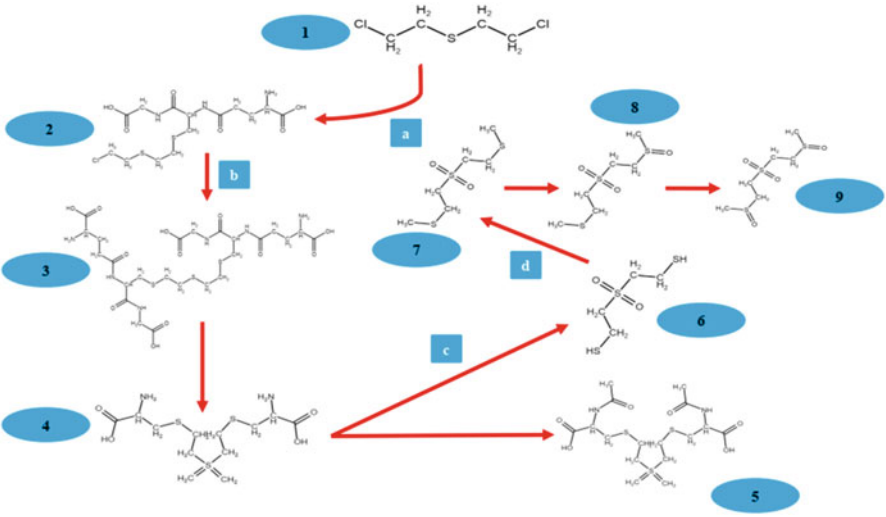

Fig. 2 Glutathione adducts of SM and major urinary metabolites of the pathway. (1, Sulfur mustard (SM); a, glutathione transferase (GSH); 2, mono-GSH-SM adduct; b, glutathione transferase (GSH); 3, di-GSH-SM adduct; 4, 1,1′ sulfonylbis-[2-S-(cysteinyl)ethane]; 5, 1,1′-sulfonylbis[2-S-(N-acetylcysteinyl)ethane] (SBSNAE); c, beta-lyase; 6, 1,1′-sulfonylbis-(ethane-2-thiol); d, S-methyl transferase; 7, 1,1′-sulfonylbis[2-(methylthio)ethane] (SBMTE); 8, 1,1′-methylsulfinyl-2-[2-(methylthio)-ethylsulfonyl]ethane (MSMTESE); 9, 1,1′-sulfonylbis[2-(methylsulfinyl)ethane] (SBMSE))

and 1,1′-methylsulfinyl-2-[2-(methylthio)-ethylsulfonyl]ethane (MSMTESE) are major urinary metabolites of this pathway. Normally, 1,1′-sulfonylbis[2-(methylthio)-ethane] (SBMTE) was not identified in urine samples of animal studies. However, it is believed that SBMTE is likely precursor of β-lyase metabolites SBMSE and MSMTESE (Barr et al. 2008; Lin et al. 2014; Xu et al. 2014).

The third metabolic pathway is the alkylation reaction of bifunctional SM with guanine and adenine residues of DNA which produces DNA adducts including monoadducts N7-[2-[(2-hydroxyethyl)thio]-ethyl]guanine (N^7-HETEG), O6-[2-[(2-hydroxyethyl)thio]ethyl]-guanine (O^6-HETEG), N3-[2-[(2-hydroxyethyl) thio]ethyl]-adenine (N^3-HETEA), and the cross-linked adduct bis[2-(guanin-7-yl) ethyl]sulfide (N^7-BisG) (Malhotra et al. 1999; Ghabili et al. 2011; Xu et al. 2014; Golime et al. 2019; Cheng et al. 2021). N^7-HETEG was 61% of the analyzed adducts, and it was followed by N^7-BisG as 17%, N^3-HETEA as 16%, and O^6-HETEG as 0.1%, respectively (Zubel et al. 2018).

Finally, the fourth metabolic pathway consists of alkylation reactions of SM with carboxyl, imidazole, thiomethyl, and sulfhydryl groups of proteins including HETE-valine (HETE-Val) adduct of hemoglobin and HETE-cysteine adduct of albumin (Malhotra et al. 1999; Xu et al. 2014; Golime et al. 2019).

Figure 3 shows third and fourth major metabolic pathways with DNA and protein adducts. Although HETE-Val protein adducts of SM have a lifetime of approximately

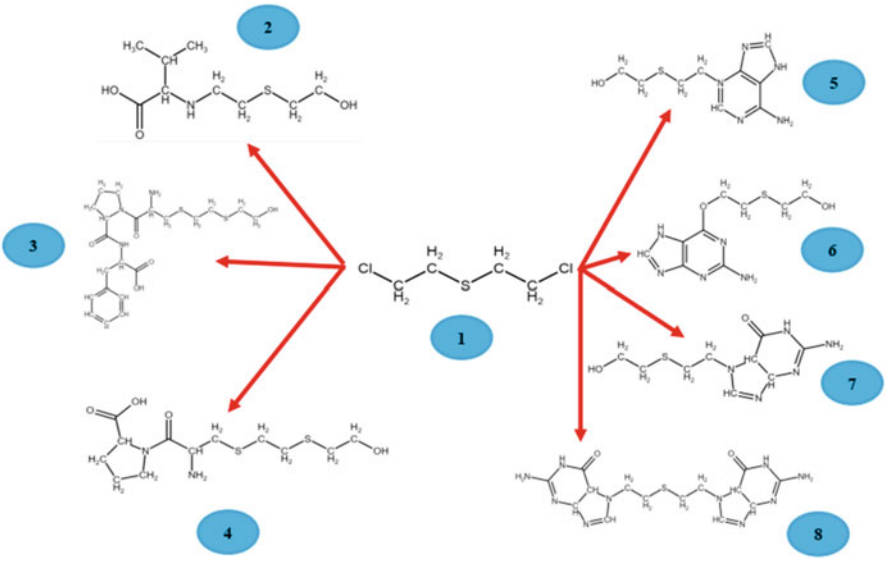

Fig. 3 DNA and protein adducts of SM and major metabolites of the pathways. (1, Sulfur mustard (SM); 2, HETE-valine adduct (HETE-Val); 3, HETE-cysteine adduct; 4, HETE-cysteine adduct; 5, N3-(2-hydroxyethylthioethyl)-2′-adenine (N^3-HETEA); 6, O6-[2-[(2-hydroxyethyl)thio]ethyl]-guanine (O^6-HETEG); 7, N7-[2-[(2-hydroxyethyl)thio]-ethyl]guanine (N^7-HETEG); 8, bis(2-ethyl-N7-guanine)thioether (N^7-BisG))

120 days in human blood, DNA adducts could be found in urine samples up to 4 weeks and in blood samples up to 14 days (Noort et al. 2002; Cheng et al. 2021).

Mechanism of Action

As mentioned before, SM is a bifunctional molecule, and it reacts with macromolecules via the reactive episulfonium ion intermediates which are readily forming covalent bonds (Xu et al. 2014; Schwenk 2018). Episulfonium ion is a highly reactive alkylating agent, and it has affinity to nucleophiles of DNA (adenine and guanine) and RNA bases. Several molecular pathways are involved in SM toxicity, but the most important molecular target of SM toxicity is deoxyribonucleic acid (DNA) (Malhotra et al. 1999). DNA alkylation, intra- or inter-strand crosslinks, and the formation of double-strand breaks are main cytotoxic effects of SM exposure. After SM-induced DNA damage, genotoxic stress lead to poly(ADP-ribose) polymerase (PARP) activation and nicotine adenine dinucleotide (NAD^+) depletion because PARPs use NAD^+ as a substrate during catalyzation of adenosine diphosphate (ADP) ribosylation which is responsible for DNA repair and gene expression. NAD^+ depletion causes impaired glycolysis and decreased intracellular adenosine triphosphate (ATP) which could lead to necrotic cell death because of impaired cell energy generation. NAD^+ depletion also increases protease synthesis which is responsible for necrosis and cell death, so it is shown that PARP modulates SM-induced cell death with different mechanisms. Cellular rupture and release of intracellular content after necrotic cell death cause to damage in surrounding cells which activates inflammatory response including interleukin (IL)-1α, IL-1β, IL-6, IL-8, granulocyte-monocyte colony-stimulating factor, monocyte-activating protein-1 (MAP-1), and TNF-α production. DNA damage network including nuclear factor-κB (NF-κB), tumor protein p53, and mitogen-activated protein kinase is also responsible for SM-induced cell death and inflammation. It was shown that SM toxicity affected Ca^{2+} channels which caused to increased level of cytosolic calcium and hemostasis imbalance of Ca^{2+}. Increased level of intracellular Ca^{2+} both activated the intrinsic mitochondrial pathways of apoptosis via calmodulin and led to induction of apoptosis markers such as p53, suppression of Bcl-2, and activation of caspase-3. Reactive nitrogen species (RNS) and reactive oxygen species (ROS) due to marked depletion of glutathione (GSH) cause not only further DNA damage but also to lead to lipid peroxidation resulting in membrane damage and cell necrosis (Kehe and Szinicz 2005; Kehe et al. 2009a; Ghabili et al. 2011; Balali-Mood and Abdollahi 2015, 69–72; Zubel et al. 2018).

Clinical Toxicology of SM Poisoning

SM has toxicological effects on the skin and ocular, respiratory, and hematological systems including bone marrow. The clinical hallmark of SM exposure is a symptom-free latency period after the contact (Sezigen et al. 2019). The onset and

severity of symptoms depend on the concentration of SM, total amount of exposed SM, total exposure time, exposure route, and specific weather conditions like high temperature and humidity (Sezigen and Kenar 2020). Eyes and the respiratory tract are more vulnerable than the skin to the exposure of gas or aerosol form of SM. Initial symptoms in the first 1 or 2 hours after the exposure are mostly burning sensation in the eyes, eye redness, sore throat, and coughing. In the first 24 hours, bilateral conjunctivitis following eyelid edema and photophobia are major ocular symptoms of SM casualties (Sezigen et al. 2019, 2020a, b; Sezigen and Kenar 2020).

SM inhalation causes sore throat, hoarseness, oropharyngeal edema, dysphagia, rhinorrhea, and nonproductive coughing in most of SM casualties. In the first 24 hours after the exposure, laryngeal spasm and pseudomembrane formation could be life-threatening complications in cases who were exposed to high-dose SM (Willems 1989; Sezigen et al. 2019; Sezigen and Kenar 2020).

Although the ocular system is the most susceptible one, the skin is the main target of SM exposure. Its lipophilic character allows SM to penetrate through the skin rapidly. First dermal contact with SM is mostly painless. Dermal SM exposure is mostly effective on moisture areas of the body including axillae and the genitals. As shown in Fig. 4, the typical presentation of dermal lesions ranges from slight edema, itching, sunburn-like erythema to vesicles, blisters, and coagulation necrosis in cases who were exposed to high concentration of the agent. The first cutaneous symptoms started mostly 2 hours after the exposure, and blisters could be seen between eighth and 24th hours. Healing of skin lesions needs more than 2 weeks. Besides brown pigmentation, hypopigmented and hyperpigmented areas could be observed on the affected skin (Willems 1989; Ganesan et al. 2010; Sezigen and Kenar 2019; Sezigen et al. 2020a).

Hematological complications including neutrophilic leukocytosis, lymphocytopenia, anemia, thrombocytopenia, neutropenia, and secondary bacterial

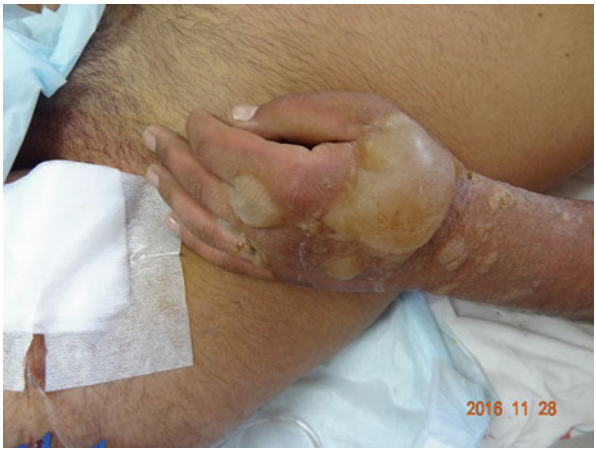

Fig. 4 Typical cutaneous symptoms including lesions ranging from erythema, vesicles, and blisters 48 hours after mustard gas exposure. (Courtesy of Dr. Sermet Sezigen)

infections due to myelosuppression are seen in victims of SM exposure (Kehe et al. 2009b; Sezigen et al. 2019, 2020a, b).

Medical Management of SM Poisoning

Although SM was used more than 100 years ago in WWI, there is still no specific antidote used for the medical management of SM casualties except symptomatic treatments in well-equipped medical centers (Thiermann et al. 2013; Sezigen and Kenar 2020). Main principles of medical management of SM casualties are specialized symptomatic treatment for ocular, respiratory, and cutaneous lesions, avoiding secondary infections, controlling fluid and electrolyte imbalance, and supporting inhalation and nutrition (Kilic et al. 2018). According to the severity of lesions, cases should be followed up in intensive care units. Hematologic parameters should be checked daily in order to follow the risk of myelosuppression in severe cases (Willems 1989; Sezigen et al. 2020a).

Removing of clothing could be an 80% decontamination of SM (Sezigen and Kenar 2020). Medical decontamination should be performed in order to prevent absorption of SM and secondary contamination of medical personnel. There is no specific decontamination product for SM exposure. A variety of industrial decontaminants which could be used against all kind of CWA exposure are also used. Decontamination teams are still using universal decontamination solutions like household bleach or water and soap solutions.

Laboratory Diagnosis of SM Poisoning through Urinary Metabolites

Bioanalysis of human biomedical samples including the urine, blood, and tissues for the verification of SM exposure is essential for diagnostic and forensic purposes. Diagnosis of SM poisoning generally focuses on typical ocular, respiratory, and cutaneous symptoms of SM exposure with a confirmed medical history of the chemical exposure without certain specific analytical capabilities (Read 2016; Sezigen and Kenar 2020).

However, early confirmation of SM exposure by detecting SM metabolites or protein/DNA adducts not only supports medical personnel for the rapid and reliable documentation of SM poisoning by means of differential diagnosis but also proves potential law violations to law enforcement forces after chemical terrorist attacks. Besides, detection and quantification of SM exposure could affect therapeutic approaches as the concentration of metabolites/adducts reflects the total absorbed SM when the treatment should be tailor-sized instead of symptomatic in cases with SM-induced myelosuppression.

Biomedical samples including the urine, blood, and tissues should be collected from the victims of SM exposure by professional trained medical personnel according to the chain of custody, and these samples should be transferred to

accredited laboratories by using secure cold-chain systems. For the verification of SM exposure, gas chromatography (GC) and liquid chromatography (LC) techniques in combination with tandem mass spectrometry (MS/MS) are mostly preferred for the off-site analysis of biomedical samples (Steinritz et al. 2016; Sezigen et al. 2019, 2020b).

Factors that define the biological fate of SM in humans include chemical stability of SM, covalent binding to macromolecules, and rates of absorption, metabolism, and excretion, and all these parameters could vary with the chemical property of SM, route of exposure, and total absorbed concentration (Black 2010).

Although protein adducts of hemoglobin and albumin have generally 6- and 3-week half-lives in humans, respectively, almost 90% of urinary metabolites are excreted within the first 72 hours after the exposure, and this main disadvantage requires working with specific and sensitive analytical methods with a low limit of detection (LOD) $<$ 1 ng/mL, lower limits of quantitation, and both positive and negative quality control samples for the verification of alleged CWA use (Black 2010; Read 2016).

As the implementing body for the CWC, OPCW is managing an international laboratories network for the verification of alleged use of CWAs, and this network is consisting of international reference laboratories that are accredited by OPCW annually through a series of confidence building exercises (John et al. 2018). OPCW-accredited laboratories are able to work with defined criteria for the unequivocal identification of CWA exposure in humans in terms of the specificity of the biomarker and the trace analytical method. Instead of unchanged SM which should be sampled early after the exposure for detecting significant levels because of its instability in aqueous solutions, these laboratories focus on identifying the principal biomarkers of SM including urinary metabolites, reaction products, and DNA or protein adducts. LOD, high specificity, and using minimum two analytical methods or two biomarkers for the confirmation and quantitation are main requirements for the ideal forensic analysis of SM exposure (Vycudilik 1987; Black 2010; Read 2016).

The initial studies which aimed to verify either alleged use or accidental exposure of SM begun in the middle of the 1980s especially when Iraq forces used SM against civilians and Iranian soldiers. Table 2 shows detailed list of detected urinary biomarkers in patients who were exposed to SM. Wils et al. (1985) analyzed urine samples of ten Iranian patients who were transferred hospitals in the Netherlands and Belgium 10 days after they were exposed to a CWA. Urine samples which were taken from chemical casualties with classical symptomatology of SM exposure were analyzed for the identification and quantitation of TDG. Although TDG concentrations varying between 3 and 140 ng/mL were found in some patients' samples, alleged use of SM against patients was not proven because TDG was not used as an unambiguous verification biomarker as quite background levels of TDG were detected in control urinary samples of the nonexposed group (Wils et al. 1985; Willems 1989; Black 2010). Wils et al. (1988) modified the analytical method and analyzed urine samples of 25 Iranian patients and 25 healthy control. They found that TDG levels of 80% of Iranian patients were higher than 95% confidence limit of the control group.

Table 2 Detailed list of detected urinary biomarkers in patients who were exposed to SM

Reference	Incident/subject	Subject code	Day after the exposure	Urinary Metabolites of SM				
				TDG	TDGO	SBMSE	MSMTESE	SBMTE
Wils et al. (1985)	Iranian patients from Utrecht hospitals	U1	**10**	+				
		U2	**10**	–				
		U3	**10**	+				
		U4	**10**	+				
		U5	**10**	+				
	Iranian patients from Ghent hospitals	G1	**10**	+				
		G2	**10**	+				
		G3	**10**	+				
		G4	**10**	+				
		G5	**10**	+				
Black and Read (1995a)	Accidental exposure	1	–	+	+	+	+	
		2	–	+	+	+	+	
Black and Read (1995b)	Iranian patients from Ghent hospitals	C1	**10**	+	+			+
		C2	**10**	+	+			+
		C3	**10**	**ND**	**ND**			+
		C4	**10**	**ND**	**ND**			+
		C5	**10**	+	+			+
	Halabja patients in London	L1	**13**	+	+			+
		L2	**13**	+	+			+
Read and Black (2004)	Halabja patients in London	L1	**13**			+	+	
		L2	**13**			+	+	
	Accidental exposure	S1	**2–3**			+	+	
		S2	**2–3**			+	+	
Barr et al. (2008)	Accidental exposure	P1	**2**	+	+			+
		P2	**2**	+	+			+

Xu et al. (2014)	Accidental exposure	P1	**3**	+	+	+	+	+
		P2	**3**	+	+	+	+	+
		P3	**3**	+	+	+	+	+
		P4	**3**	+	+	+	+	+
Steinritz et al. (2016)	Accidental exposure	1	**3**			+	+	
		2	**3**			+	+	
		3	**3**			+	+	
Sezigen et al. (2019)	Syrian patients	P1	**2**					+
		P2	**2**					+
		P3	**2**					**ND**
		P4	**2**					**ND**
Sezigen et al. (2020b)	Syrian victims of chemical terrorism	P1	**30 h**					+
		P2	**30 h**					+
		P3	**30 h**					+
		P4	**30 h**					+
		P5	**30 h**					+
		P6	**30 h**					+
		P7	**30 h**					+
		P8	**30 h**					+
		P9	**30 h**					–
		P10	**30 h**					–
		P11	**30 h**					–
		P12	**30 h**					–
		P13	**30 h**					–

[a]h, hour
[b]ND, not determined due to insufficient urine sample

Black et al. (1992) administered SM to the rats through intraperitoneal route in order to identify biological fate of SM, and they isolated urinary metabolites including hydrolysis product TDGO, glutathione conjugate SBSNAE, and β-lyase metabolites SBMSE and MSMTESE. Then Black and Read (1995a) performed quantification of SM metabolites including TDG, TDGO, SBMTE, and MSMTESE in urine samples of two patients who were accidentally exposed to SM. It was concluded that TDGO was not an ideal biological marker of SM poisoning as it was also found at low concentrations in normal human urine like TDG. On the other hand, it was also reported that β-lyase metabolites were unequivocal diagnostic biomarkers of SM exposure as they were not found in healthy subjects. Black and Read (1995b) also analyzed urinary metabolites of SM derived from hydrolysis and the β-lyase pathway in seven casualties who were exposed to SM by using an improved methodology in GC-MS/MS. Both SBMSE and MSMTESE were reduced to SBMTE and TDGO to TDG by using acidic titanium trichloride. It was reported that levels of TDG and TDGO were higher than the levels of β-lyase metabolites because of slow release of TDG from acidic sites on proteins and nucleotides.

When CWC entered into force in 1997, lots of international research laboratories begun to focus on analysis of biomedical samples because alleged use of SM in the Iran-Iraq War showed that more specific and sensitive analytical methods should be developed for the on-site and off-site verification of alleged use of CWAs. At the beginning of the 2000s, LC-MS systems which provided full chemical specificity including inducible compound fragmentation and accurate measurement of mass to charge ratios (m/z) had begun to be used in order to achieve lower detection limits for the analysis of biomedical samples (Black 2010; Zubel et al. 2018).

However, SM itself had never been investigated so much as a useful biomarker because of its residence time in the blood less than 90 min (Zubel et al. 2018).

Read and Black (2004) developed a rapid LC-MS/MS method for the analysis of urinary β-lyase metabolites with electrospray ionization (ESI) technique. This method both detected β-lyase metabolites separately without converting them to a less polar metabolite SBMTE. They analyzed urine samples of four SM casualties and they detected both SBMSE and MSMTESE in urinary samples separately.

Boyer et al. (2004) developed a method for the detection of TDG, TDGO, SBMSE, MSMTESE, and SBMTE by GC-MS/MS using isotope dilution. In another study, this method was used for the analysis of urine samples of two casualties who were exposed to SM accidentally. TDG, TDGO, and SBMTE were detected in all samples, and it was reported that β-lyase metabolites were especially found at their highest concentration 2 days after the exposure and the half-life of SBMTE in urine samples was less than 1 day (Barr et al. 2008). Lin et al. (2014) also concluded that maximal excretion of urinary β-lyase metabolites was detected in the first 40 hours after the exposure and a rapid elimination pathway due to the reaction with glutathione caused urinary excretion of 99% of total β-lyase metabolites in the first 7 days after SM exposure.

Steinritz et al. (2016) analyzed urine samples of three civilians who were exposed to SM accidentally by using LC-ESI MS/MS method based on a previous study.

They detected both SBMSE and MSMTESE in three patients 3 days after the exposure.

Xu et al. (2014) collected blood, urine, and blister exudates from four civilians who were admitted to the hospital with blister formations after contact to unknown chemical in a barrel at a construction site. They performed all analysis by using both GC-MS/MS and LC-MS/MS for the detection of hydrolysis/oxidation products TDG and TDGO in urine samples; glutathione-derived metabolites SBSNAE, SBMTE, MSMTESE, and SBMSE in urine, blood, and blister exudates; SM-DNA adducts N^7-HETEG, O^6-HETEG, N^3-HETEA, and Bis-G in urine and blood samples; and SM protein adduct HETE-Val in blood samples. It was reported that TDGO levels were higher than TDG levels in urinary samples of patients and both TDG and TDGO were in higher concentrations 3–4 days after SM exposure and decreased from 5 to 7 days. On the other hand, detection window of TDG and TDGO in the blood was the initial 48 hours after SM exposure. It was also noted that β-lyase metabolites had a detection window with a sharp decline between fourth and fifth days after the exposure in urine samples. Also, comparing with SBSNAE and SBMTE, SBMSE and MSMTESE were much more identified in urine, blood, and exudate samples. Urinary SM-DNA adducts including N^7-HETEG, Bis-G, N^3-HETEA, and O^6-HETEG were, respectively, detected in the first 32 days after the exposure with a maximum peak on the first week in urine samples. SM-DNA adducts were found in detectable concentrations in the first 2 weeks after the exposure in blood samples. There was a correlation found between the concentrations of β-lyase metabolites and SM-DNA adducts and severity of the clinical pictures in victims of SM exposure. Finally, HETE-Val exhibited much longer detection window so it was detected over 90 days after the exposure in blood samples, and it was concluded that HETE-Val was a valuable biomarker for the retroverification of SM exposure over 3 months and it could be used for the following of clinical treatment (Xu et al. 2014; Zubel et al. 2018).

Alleged use of SM against civilians by non-state actors in Syria during the ongoing civil war after 2015 was proven with several studies which analyzed biomedical samples of chemical casualties. Sezigen et al. (2019) performed quantitative analysis of SBMTE by using a GC-MS/MS method which is based on a previous study in urine samples of a Syrian family who was exposed to SM in 2015 (Eyison et al. 2019). Urine samples were collected 48 hours after the exposure. Although they found high concentration of SBMTE in urine samples of parents (Patient 1 and Patient 2), levels of SBMTE were not detected in urine samples of their children (Patients 3 and 4) due to insufficient amount of urine samples.

John et al. (2019) detected four peptide biomarkers of SM from serum albumin in blood samples of four chemical casualties (total of seven casualties) who had typical symptoms of percutaneous SM exposure. Blood samples were taken 15 days after the exposure.

Sezigen et al. (2020b) analyzed urine samples of another group of SM casualties. Urine samples of 13 patients, who were exposed to SM through a SM-filled trapped improvised explosive device, were collected 30 hours after SM exposure. The samples were analyzed for the detection of SBMTE by using GC-MS/MS.

SBMTE was detected in various levels in the first seven patients who were the nearest victims to the detonation. It was concluded that β-lyase metabolite SBMTE was an unambiguous biomarker for the verification of SM exposure and there was a positive correlation between urinary SBMTE levels and severity of clinical presentation in SM-exposed chemical casualties (Sezigen and Kenar 2020; Sezigen et al. 2020b).

Besides off-site detection of SM exposure by using complicated analysis systems, point-of-care (POC) testing is also a rapid diagnostic method for the verification of SM exposure. Ye et al. (2021) developed a test strip for the detection of TDG in urine samples with a LOD level of 0.23 ng/ml. It was reported that POC tests could reduce time and costs for the collection, storage, and transportation of biomedical samples. Besides, rapid preliminary results could support further diagnosis of SM exposure in patients with typical symptoms of SM poisoning.

Applications to Prognosis

Major urinary biomarkers which are used for the verification of SM exposure in humans and development of bioanalysis techniques have been reviewed in this chapter through important studies which improved our knowledge about the toxicokinetics and dynamics of SM exposure.

Although OPCW (2021c) reported that 193 states which signed the Chemical Weapons Convention destroyed 98% of the declared chemical weapons stockpiles through national chemical weapon demilitarization programs, risk of alleged use of CWAs against mostly civilians by rogue states or non-state actors is still present. When strategic-level policies that aim to prevent the proliferation of chemical weapons fail and a chemical terrorist attack or assassination occurs, by means of medical chemical defense, the most important step is rapid detection and identification of the CWA by using both environmental and biomedical samples including the blood, urine, and tissues.

Major urinary metabolites are identified for SM, and SM is the most previously weaponized CWC Schedule 1 CWA due to its easy chemical synthesis based on basic chemicals. In the presence of a core bioanalytical capability for CWAs, urinary metabolites have great importance for the further diagnosis of SM exposure and following up chemical casualties especially in intensive care units (Black 2010; Xu et al. 2014; Zubel et al. 2018; Sezigen and Kenar 2020). Beside medical applications, forensic detection of biomedical samples supports clear proof for the alleged use of CWAs (Mangerich and Esser 2014). Despite their limitation for the retrospective detection of SM exposure because of their rapid elimination, readily accessible urinary metabolites including β-lyase metabolites and some DNA adducts have proved themselves as unequivocal biomarkers of SM poisoning (Balali-Mood and Hefazi 2005; Sezigen et al. 2020b). However, it should be noted that total amount of DNA adducts is less than total amount of protein adducts (Zubel et al. 2018). Recent technical development in more selective and sensitive MS methods and advancement of proteomic techniques are facilitating the identification of specific SM

adducts not only in urine samples but also in complex matrices like blood or blister exudates. From this perspective, development of new sampling and sample preparation/pretreatment procedures, more sensitive bioanalytical techniques for DNA and protein adducts with the use of isotopically stable suitable internal standards, and rapid point-of-care diagnostics kits could be also expected (Black 2010; Read 2016). As a result, quantitative bioanalysis of urinary metabolites of SM is required not only for proving alleged use of SM but also for a better understanding of biological fate of SM exposure.

Mini-Dictionary of Terms

Alleged use. An action that has been stated but has not been confirmed.

β-lyase metabolites. Unequivocal diagnostic biomarkers of SM exposure including SBMSE, MSMTESE, and SBMTE.

Bioanalysis. Detection and identification of metabolites or drugs in biomedical samples by using instrumental techniques.

Biomedical samples. Urine, blood, and tissue samples that are taken for diagnostic or forensic analysis.

Chemical casualty. Patient who is exposed to either chemical warfare agents or toxic industrial chemicals after an accident or a chemical attack.

Chemical terrorism. Act of violence on vulnerable targets – especially civilians – by using chemical warfare agents or toxic industrial chemicals.

Exposure. Being contaminated by either chemical, biological, or radiological agents through either the eyes, the skin, the respiratory tract, or the gastrointestinal system.

Urinary metabolite. The biomarkers that are excreted to the urine mostly within the first 72 hours after the exposure.

Vesicants. A group of chemical warfare agents including sulfur mustard, nitrogen mustards, and lewisites that mostly affect the skin with chemical burns.

Key Facts of Biomarkers of Sulfur Mustard (Mustard Gas) in Urine

- Sulfur mustard is an alkylating vesicant and a banned chemical warfare agent.
- It was first used near Ypres, Belgium, on July 12 and 13, 1917, by German forces against British and Canadian forces.
- The eyes, the respiratory tract, and the skin are major routes of SM exposure.
- SM has toxicological effects on the skin and ocular, respiratory, and hematological systems including bone marrow.
- There is still no specific antidote used for the medical management of SM casualties except symptomatic treatments in well-equipped medical centers.
- After the alleged use of SM, the verification of the exposure by detecting the related biomarkers is essential for diagnostic and forensic purposes.

- The major biomarkers of SM are urinary metabolites including hydrolysis products and glutathione adducts, DNA, or protein adducts.
- Urinary metabolites including β-lyase metabolites and some DNA adducts have proved themselves as unequivocal biomarkers of SM poisoning.

Summary Points

- Sulfur mustard (SM) is a vesicant chemical warfare agent and it mainly affects the eyes, the respiratory tract, and the skin.
- It is absorbed through the ocular, respiratory, and cutaneous routes.
- It has cytotoxic, teratogenic, mutagenic, and carcinogenic effects on human beings.
- Hydrolysis/oxidation products, β-lyase metabolites, DNA, and protein adducts are main metabolites of SM.
- Bioanalysis of SM metabolites in biomedical samples including the blood, urine, and exudates is essential for the verification of SM exposure.
- Urinary β-lyase metabolites including SBMSE, MSMTESE, SBMTE, and DNA adducts including N^7-HETEG, O^6-HETEG, N^3-HETEA, and N^7-BisG are unambiguous biomarkers of SM poisoning.
- Quantitative bioanalysis of urinary metabolites of SM is required not only for proving alleged use of SM but also for a better understanding of biological fate of SM exposure.

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Biomarkers of Cardiopulmonary Injury and Impact of Bromine Toxicity

33

Ginette Bordcoch and Juan Xavier Masjoan Juncos

Contents

Abstract

Bromine is a highly oxidizing agent used in many industries. Therefore, it needs to be stockpiled and transported in significant quantities, making the risk for accidental and deliberate exposure a scenario that must be considered. In bromine exposure, while there are involvement and injury to external tissues such as the eyes and skin, its main point of entry to the body is through the respiratory system. Bromine exposure can cause different injury levels, from mild irritations to death, depending on the concentration of bromine and the duration of the exposure. Bromine affects the respiratory and circulatory systems causing acute respiratory distress syndrome and cardiocirculatory collapse while mediating cellular death. This injury can be assessed through molecular markers of inflammation, oxidative stress, cellular death, and function measurements such as

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spirometry and echocardiography. Simultaneously, imaging analysis can reveal the extent of inflammation in the pulmonary tissue. A better understanding of the injury mechanism leads to better use of clinically available tools.

Keywords

Bromine · ARDS · Cardiocirculatory collapse · Halogen · Brominated reactants · Toxicity · Systemic inflammation · Oxidative stress · Prognosis · Hypoxia · Multi-organ failure

Abbreviations

Br_2	Bromine
Br-FA	Brominated fatty acids
Br-FAD	Brominated fatty aldehydes
cTnI	Cardiac troponin I
eNOS	Endothelial nitric oxide synthetase
GFAP	Glial fibrillary acidic protein
hFABP	Heart fatty acid-binding protein
IDLH	Immediately dangerous to life and health
IL-6	Interleukin-6
LDH	Lactate dehydrogenase
NT-proBNP	N-terminal pro-brain natriuretic peptide
ppm	Parts per million
SERCA	Smooth endoplasmic reticulum calcium ATPase

Introduction

As the uses of Br_2 and its demand continue to expand, we are faced with an increased stockpile and transport of this highly reactive agent. This brings along an increased risk for deliberate and accidental release and subsequent exposure to this chemical agent. Br_2 is a highly oxidizing halogen whose IDLH concentration is much lower than chlorine and ammonia, thus making it far more toxic than both (Makarovsky et al. 2007; O.M.J. Kasilo 1999). Halogen exposure has been reported in isolated events for decades. However, most known injury mechanisms for different organs and tissues have been reported in recent years. Most of the research aimed to elucidate the mechanisms of injury has focused on the impact that Br_2 toxicity has on the lungs and heart. Presently, many of the injury mechanisms for other organs remain unknown. The development of new biomarkers specific for Br_2 exposure-induced injury grants us valuable tools to help us better understand the extent of injury and prognosis in the event of Br_2 exposure. Furthermore, because of the nature of the exposure settings, it would be safe to assume that in the event of a release of Br_2, it would lead to mass exposure in the area where this was to occur. Knowledge of the significance of individual biomarkers in mass exposure events presents an invaluable tool for the triage process.

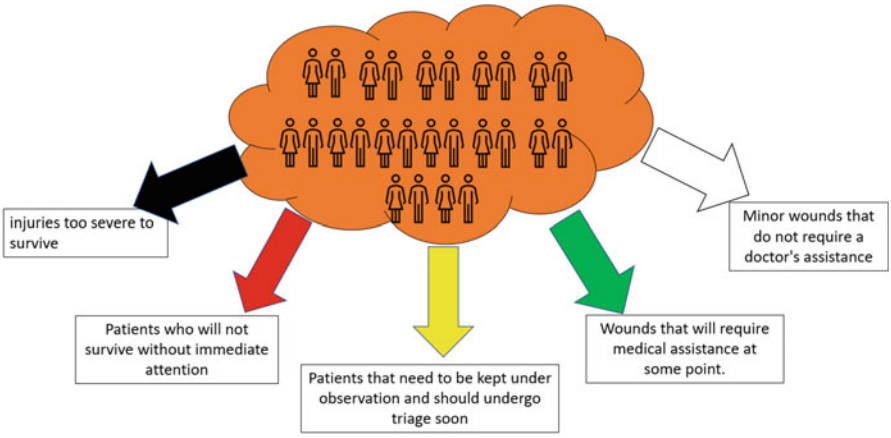

Fig. 1 Mass exposure event, are we ready for this triage? One of the dangers of Br2 exposure is the lack of proper tools to assess the severity of injuries, as the state of the patient's health continues to worsen in the hours following exposure. A better understanding of diagnostic tools at our disposal can increase the quality with which this process is carried out.

Since exposure events are rare, the use of previously established and clinically available biomarkers of end-organ injury of known affected systems has played a prominent role in assessing Br_2-induced injury. The expediency with which treatment is administered in these cases can vastly increase the odds for a more favorable outcome. Molecular, physiologic, histologic, or radiographic biological markers are required to assess the extent and severity of injury. The availability of these methods would allow for a more accurate triage process should there be a mass exposure event (Fig. 1). The use of previously established tests such as cardiac injury markers used in acute myocardial infarction, central nervous system injury markers, and pulmonary injury markers, among others, provides tools for immediate application. This chapter aims to provide a greater understanding of the biomarkers available for assessing Br_2-induced injury and the meaning behind the presence of each biomarker.

Mechanisms, Presentation, and Biomarkers of Respiratory Bromine Toxicity

Mechanisms of Br_2 toxicity on the respiratory system. The main access point of Br_2 into the body is the respiratory system. There is a reaction between Br_2 and its hydrolysis product, hypobromous acid, when coming in contact with the epithelial cell lining. Both are strong oxidants which deplete antioxidants present in tissue. When no more antioxidants are available, Br_2 reacts with fatty contents and generates brominated fatty acids (Br-FA) and brominated fatty aldehydes (Br-FAD), which are then released into the circulation and reach other tissues through systemic

circulation (Duerr et al. 2018) (Fig. 2). Br_2-exposed animals have been shown to have elevated heme concentration in plasma, lung tissue, and bronchoalveolar lavage fluid. Alterations in heme concentration result from significant hemolysis due to increased red blood cell fragility given by the reaction between the cellular membrane of red blood cells and Br_2. Increased concentration of free heme leads to further inflammation as it causes additional oxidative stress and cellular damage. At the same time, the scavenging of free heme has shown promise as a possible therapeutic alternative to treat these injuries (Aggarwal et al. 2016; Lam et al. 2016). In the lungs, the oxidative stress caused by Br2 and its byproducts (bromine's reaction with other components of extracellular fluid and cellular components such as heme, Br-FA, Br-FAD, among others) causes pulmonary congestion with increased edema that compromises the air space within the alveoli and bronchi. There is also an increase in white blood cells in the pulmonary interstitium (Lambert et al. 2017), leading to acute respiratory distress syndrome, apoptosis, pulmonary edema, and reactive airway dysfunction. The evolution of this injury resolves into pulmonary fibrosis as a chronic consequence of the injury (Zhou et al. 2018).

Presentation of Br2 toxicity on the respiratory system. Upon entry to the upper respiratory airways, Br_2 causes irritation, inflammation, and injury to mucous

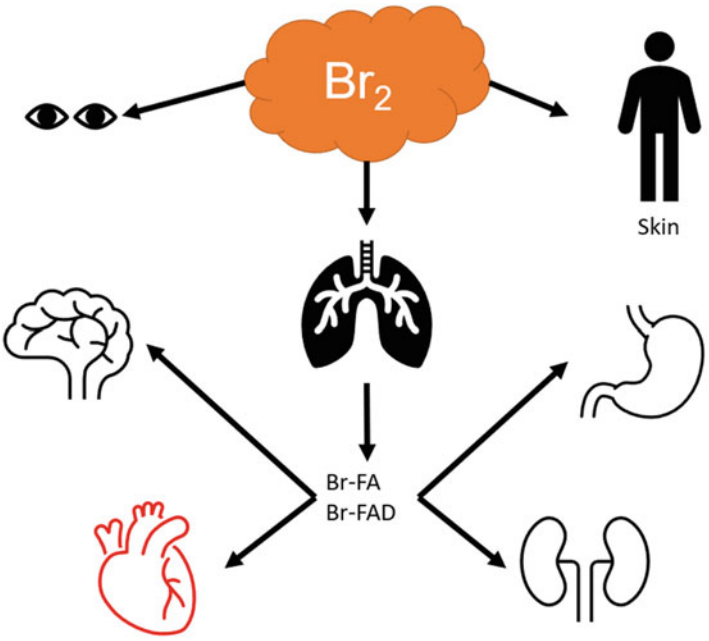

Fig. 2 Br2 distribution through the body. Only the eyes, skin, oral cavity, and airways come in contact with the halogen in its pure form. Once it is in the lungs, it reacts with blood components as vascular integrity is compromised and forms Br-FA and Br-FAD. In this form, it is then transported in the blood through systemic circulation to the different organs. This allows for it to easily cross the blood-brain barrier.

membranes (Morabia et al. 1988). The most common presentation is rhinorrhea, epistaxis, laryngeal edema, and tracheobronchitis (Liubchenko and Alekseeva 1991; Makarovsky et al. 2007). In the lower respiratory airways and the lungs, it has been shown that Br_2 exposure causes dyspnea, cough, choking, wheezing, immediate or delayed bronchoconstriction, acute lung injury, laryngeal and pulmonary edema, asthma, chemical pneumonitis, bronchiolitis, and mediastinal emphysema in the acute stages (Makarovsky et al. 2007; Inagaki et al. 2005). The inflammatory response that happens in the days immediately following Br_2 exposure leads to respiratory failure and subsequent hypoxemia (Lossos et al. 1990). Animal models have shown that when pregnant animals are subjected to Br_2 exposure, the offspring suffers a significant impairment of their pulmonary development (Addis et al. 2020b; Jilling et al. 2018) (Fig. 3). In chronic cases, bronchopulmonary dysplasia, quasi-static lung compliance, toxic bronchitis, damage to the lower respiratory tract, esophageal and pyloric stenosis, diffuse interstitial pulmonary fibrosis, emphysema, and airway hyper-reactivity have been reported as expected presentations (Liubchenko and Alekseeva 1991; Makarovsky et al. 2007).

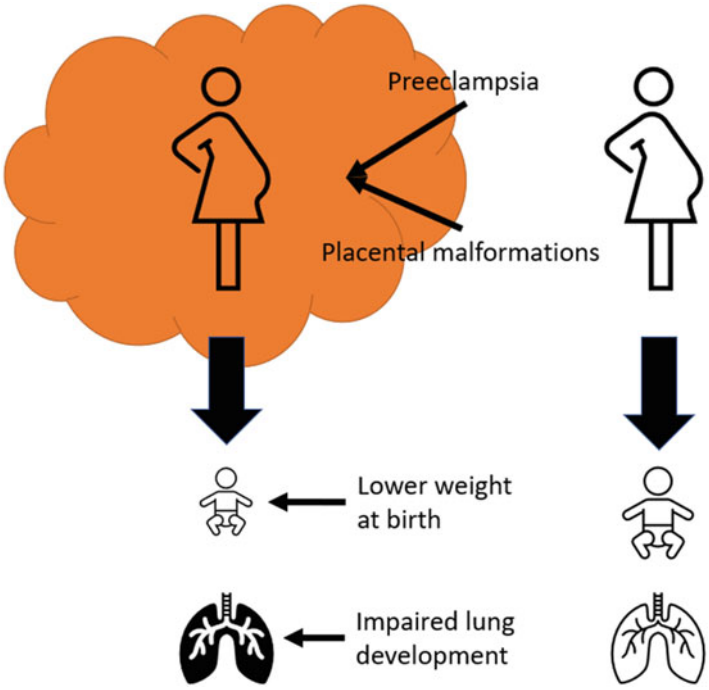

Fig. 3 Impact of Br2 exposure on pregnancy. Bromine exposure on pregnant animals suggests a significant increase in the number of risks a pregnant woman would encounter when in a situation of bromine exposure. Besides the effects Br_2 exposure has in the general population, pregnant women are also at risk of preeclampsia, placental malformations, decreased weight at birth, and impaired development of the fetus's lungs.

Biomarkers of Br2 toxicity on the respiratory system and applications to other diseases. Following bromine exposure, markers for respiratory injury can be found both in the blood and in bronchoalveolar lavage fluid. Here we can find an increased percentage of cells, heme, LDH, and protein content after Br_2 exposure (Aggarwal et al. 2016). In the blood, changes can be observed in arterial blood gases analysis, such as decreased pH, PaO_2, and increased $PaCO_2$, indicating respiratory acidosis resulting from pulmonary injury (Lambert et al. 2017). Imaging studies such as chest X-ray or CT scans can also provide evidence of the extent of the pulmonary injury denoting an essential inflammatory component to this injury (Makarovsky et al. 2007). Physiological studies of lung capacity and reactivity would serve as an essential tool for monitoring a patient's progress following Br_2 exposure. These measurements are carried out routinely in most clinical laboratories, and the translational application from shared mechanisms proven in experimental models presents an opportunity to use already available diagnostic tools. It is worth mentioning that the collection of samples for arterial blood gasses requires a much simpler procedure, though it is not as accurate when assessing injury to a single organ as injury to other organs may alter some of these values. While Br_2 has not been demonstrated to be carcinogenic, a 2.6-fold increase in the expression of squamous cell carcinoma antigen recognized by T cells 3 has been reported in the lungs 24 h after exposure in animal models (Addis et al. 2020a). A summary of these markers is provided in Table 1.

Table 1 Biomarkers of Br_2-induced lung injury. Biomarkers available to assess the impact of Br_2 exposure on the lung can be divided into markers of injury, such as percentage of cells and protein content in bronchoalveolar lavage fluid indicators of inflammation such as chest X-rays and computerized tomography (CT), and markers of pulmonary function such as arterial gas results and spirometry to measure lung reactivity and total lung capacity.

Biomarkers	Methods	Other clinical applications
Percentage of cells	Microscopy or cell counter	Pumonary toxic injury due to broncheoaspiration, toxic.
Protein content	Absorbance	Cancer, toxic pneumonitis, etc.
PH	Arterial Blood Gas analyzer	Acute respiratory distress syndrome.
PaO_2	Arterial Blood Gas analyzer	Acute respiratory distress syndrome.
$PaCO_2$	Arterial Blood Gas analyzer	Acute respiratory distress syndrome.
Imaging studies	X-Ray or CT scan	Pulmonary inflammation due to infection, cancer, toxic pneumonitis, etc.
TLC	Spirometry	Asthma, bronchiectasia, etc.
Lung reactivity	Spirometry	Asthma, bronchiectasia, etc.

Mechanisms, Presentation, and Biomarkers of Cardiac Bromine Toxicity

Mechanisms of Br_2 toxicity on the heart. Bromination occurs both due to the high reactivity of Br_2 and activity by neutrophils and eosinophils. In the heart, Br_2 arrives as Br-FA and Br-FAD, it reacts with several cellular structures and impairs their function. One key molecule affected by bromination is SERCA, the primary calcium transporter from the cytoplasm back into the endoplasmic reticulum, one of the critical cardiac muscle processes during diastole (Ahmad et al. 2019). A reduced SERCA activity is one of the hallmarks of heart failure. Br_2 has been shown to cause an exacerbated in reduced SERCA models, adding to these subjects pre-existing cardiac injury when compared to SERCA sufficient subjects (Masjoan Juncos JX et al. 2020b). Impaired ion transport across membranes alters membrane potentials causing changes in heart rate and leading to arrhythmias. Increased cytosolic calcium impairs relaxation, and this leads to diastolic dysfunction. Immediately following Br_2 exposure, there is a cardiocirculatory collapse and, in some cases, cardiogenic shock and death. After an initial decrease in cardiac rhythm, there is an increase in contractility and systemic vascular resistance, resulting in increased mean arterial pressure (Ahmad et al. 2019; Masjoan Juncos et al. 2021). The increase in blood pressure is made more significant due to the decreased bioavailability of nitric oxide that follows Br_2 exposure due to decreased eNOS expression in the vasculature, thus impairing vascular relaxation (Addis et al. 2021a). Decreased SERCA activity also leads to a cytosolic calcium overload, activating calcium-dependent proteases such as calpains. Calpain activation leads to degradation of cellular structures and mitochondrial injury. Among the cellular structures being degraded are the myofilaments responsible for muscular contraction. Myofibrillar degradation results in loss of the cardiac ultrastructural composition of cardiac muscle, impairing its proper function (Zaky et al. 2015; Ahmad et al. 2019). Compensatory mechanisms by the cardiac muscle are not sustainable long term, and the cardiac muscle continues to degenerate while function progresses towards chronic heart failure. The injured muscle is subjected to continued oxidative stress and remodeling, with increased interstitial fibrosis, while SERCA activity remains deficient even in the long term (Masjoan Juncos et al. 2020).

Presentation of Br_2 toxicity on the cardiovascular system. After incidents of Br_2 exposure, subjects have presented hypoxemia, hypercapnia, sinus tachycardia, cardiac arrhythmias, chest pain, and cardiac arrest. In chronic models, there is hypotension due to myocardial degeneration (Makarovsky et al. 2007; Woolf and Shannon 1999). Immediately following Br_2 exposure, there is a decrease in heart rate and oxygen concentration in the blood (Masjoan Juncos et al. 2021).

Biomarkers of Br2 toxicity on the cardiovascular system and applications to other diseases. Functional changes accompanying injury can be assessed by imaging analysis such as Doppler echocardiography studies. Here, the hypercontractility becomes evident by an increase in circumferential fiber shortening velocity,

+dp/dt, and ejection fraction, as well as a decrease in end-systolic diameter and volume. This analysis can also obtain evidence of diastolic dysfunction with a decreased E/A ratio, end-diastolic diameter, and volume (Ahmad et al. 2019). Molecular injury markers regularly used in the clinical setting can determine the different aspects of cardiac injury. When myofibrillar degeneration occurs in the heart, cTnI is released into the blood, the concentration of this biomarker is elevated 3 h after exposure, and remains elevated as the heart continuously undergoes structural remodeling while its function continues to decay. When the heart is subjected to stress, there is an increase in NT-proBNP in the blood, though peak concentration is not reached until 24 h postexposure. As the heart continues to be subjected to stress on its way to heart failure, NT-proBNP remains elevated (Fan et al. 2017). The loss of cell-to-cell adhesion, hFABP is released into the blood. hFABP concentration peaks at 3 h postexposure and disappears by 12 h after exposure, though in cases with a previously diseased heart, Br_2 exposure causes it to remain elevated 24 h postexposure just as it does in acute cardiac injury models (Masjoan Juncos JX et al. 2020a; Ahmad et al. 2019; Masjoan Juncos et al. 2020b; Bivona et al. 2018). The increase of these molecular markers not only evidences the ongoing injury but has also been demonstrated to be vital prognostic tools for the evolution of heart disease, as elevated values of NT-proBNP and cTnI after an acute injury have been linked to worse outcomes in the long term (Clark et al. 2019). Improvement in the values found in blood of these cardiac injury markers in experimental models have been reported to lead to better outcomes (J.X. Masjoan Juncos et al. 2021). A summary of these markers is provided in Table 2.

Table 2 Biomarkers of Br_2-induced cardiac injury. After Br2 exposure, changes in cardiac function can be assessed by echocardiography, where ejection fraction (EF) end-systolic volume, end-diastolic volume, and E/A ratio can be quantified. Injury and stress markers already widely used, such as cTnI, hFABP, and NT-proBNP, are already available and used in the clinical setting (Keller et al. 2011; Li et al. 2010; Rusconi et al. 2010)

Biomarkers	Methods	Other clinical applications
Hypercontractility	Echocardiogram	HF, valvular disease
EF	Echocardiogram	HF, valvular disease
ESV and diameter	Echocardiogram	HF, valvular disease
E/A ratio	Echocardiogram	HF, valvular disease
EDV and diameter	Echocardiogram	HF, valvular disease
CTnl	Chemiluminescent microparticle immunoassay (CMIA)	Acute coronary syndrome (ACS), particularly with inconclusive ECG
NT proBNP	Rapid immunochromatographic method	Diagnosis of acute myocardial infarction, monitoring heart failure, cardiac risk and outcome evaluation.
H-FABP	Rapid immunochromatographic method	Diagnosis of acute myocardial infarction, monitoring heart failure, cardiac risk and outcome evaluation.

Mechanisms, Presentation, and Biomarkers of Bromine Toxicity on External Tissues

Mechanism of Br_2 toxicity on external tissues. The severity of skin lesions varies depending on the extent of the exposure ranging from first-degree to third-degree burns, as reported in several cases (Inagaki et al. 2005; Carel et al. 1992; Lossos et al. 1990). The concentration of Br_2 and the length of exposure have been established as the main determinants of the extent and severity of injuries, as lower concentrations or shorter times cannot injure deep dermal tissue (Snider et al. 2014; Eliaz et al. 1998). Once the skin is exposed to Br_2, there are immediate changes in expression on several pathways involved in inflammation and wound healing, such as interleukin-1β, interleukin-2 receptor gamma chain, plasminogen activator inhibitor-1, and heme oxygenase-1, among others. These pathways have been identified as possible therapeutic targets following Br_2 exposure (Rogers et al. 2011).

Presentation of Br2 toxicity on external tissues. Upon direct contact with Br_2, the moisture on the skin tissue reacts and causes skin lesions to appear as redness, pain with brown discoloration, measles-like rash, vesicles, blisters, discharging pustules, furuncles, deep-seated ulcers, and chemical burns. Skin lesions progressed to dermatitis, slow-healing ulcers, bromoderma tuberosum, alopecia, and an acneiform papular eruption of the face and hands. When the eyes are subjected to Br_2 exposure, they present with increased lacrimation, irritation, pain, blurred vision, conjunctivitis, erosion of the sclera, photophobia, and blepharospasm. These presentations also apply to the chronic stages of injury (Makarovsky et al. 2007).

Biomarkers of Br2 toxicity on external tissues. After Br_2 exposure, the external organ injury needs to be assessed accurately to administer timely and adequate treatment through a thorough physical examination, as, currently, no biomarkers are available that provide a better alternative. Though not timely, the histological analysis of the skin and depth of the injury could provide a significant prognosis tool (Snider et al. 2014).

Systemic Mechanisms, Presentation, and Biomarkers of Bromine Toxicity

Systemic mechanism of Br2 toxicity. Other organs are also affected by brominated reactants Br-FA and Br-FAD. Brominated lipids can freely cross the blood-brain barrier and come in contact with the central nervous system. Immediately following exposure, there is a decrease in the concentration of catecholamines in the blood. This helps explain the previously mentioned decrease in heart rate as a component of circulatory collapse since the compensating mechanism by the central nervous system is not present (Ahmad et al. 2019). There is a decrease in tryptophan hydroxylase expression following Br_2 exposure, while there is an increase in dopamine due to edema and increased oxidative stress in the central nervous system (Shakil et al. 2021). The presence of Br_2 in the systemic circulation triggers a

systemic inflammatory response with increased white blood cells and cytokines (Masjoan Juncos et al. 2020; Aggarwal et al. 2016). When Br_2 interacts with red blood cells, there is an increased fragility to these cells, leading to hemolysis and an increase in free heme in plasma (Aggarwal et al. 2016). In animal models during pregnancy, Br_2 causes placental abnormalities and symptoms that resemble pre-eclampsia. Damage to the placenta is most likely due to long-acting mediators such as Br-FA and Br-FAD, increased cytokines, and decreased eNOS (Addis et al. 2021b; Lambert et al. 2017). Some indicators suggest injury in several organs, such as the gastrointestinal system, the liver, and the kidneys, although the information regarding the mechanisms for said injury is not yet available. It is possible for Br_2-induced injury to share oxidative and apoptotic features as previously described in other organs and be exacerbated by organ failures such as hypoperfusion due to cardiocirculatory collapse, hypoxemia, and the respiratory acidosis that results from respiratory failure in these cases (Makarovsky et al. 2007; Liubchenko and Alekseeva 1991). After Br_2 exposure, there have also been reports of decreased spermatogenesis, leading to loss of fertility (Potashnik et al. 1992).

Systemic presentation of Br2 toxicity. Central nervous system Br_2-induced toxicity presents with ataxia, slurred speech, tremor, nausea, vomiting, lethargy, vertigo, visual disturbances, unsteadiness, headaches, impaired memory and concentration, disorientation, hallucinations, delusions, psychotic behavior, stupor, and coma. While in chronic cases, there are reports of irritability, anorexia, and headaches (Makarovsky et al. 2007). There is increased excitability and aggression in animal models, starting at 24 h post-Br_2 exposure (Shakil et al. 2021). In the gastrointestinal system, Br2 toxicity presents with burns in the mouth, throat, and stomach, brown discoloration and corrosion of the tongue and mucous membranes, sore throat, vomiting, abdominal spasm, severe gastroenteritis with possible ulceration or perforation, and prostration (Makarovsky et al. 2007).

Systemic biomarkers of Br2 toxicity and applications to other diseases. In order to assess Br_2-induced toxicity in the central nervous system, molecular markers such as GFAP have been repurposed from ischemic and traumatic brain injury models, with increased plasmatic concentrations in cases of injury (Lv et al. 2015; Shakil et al. 2021; Zhang et al. 2014; Yue et al. 2020). In the blood, we can observe increased concentrations of Br-FA and Br-FAD immediately after exposure to Br_2, remaining elevated well past 24 h following exposure. These have been reported to be measured by liquid chromatography/mass spectrometry after Dole extraction, and their concentration has been proven to correlate to functional changes and cardiac and pulmonary injury markers. Optimization of these techniques could grant a fast and accurate tool to determine the level of exposure in a mass exposure event (Juncos et al. 2020; Wacker et al. 2013). Serum Br_2 has also been measured using X-ray fluorescence, though this has only been used in cases of plant worker's chronic exposures. However, these measurements are not clinically useful due to the complexity of the method and the time it requires to obtain the results (Eldan et al. 1996). Laboratory findings in reports of Br_2 exposure include leukocytosis, moderate hypoglycemia or altered blood sugar curves, hypercholesterolemia, reduction in

total bilirubin, decreased hemoglobin concentration, increased erythrocyte sedimentation rate, and elevation of liver-related enzymes (Liubchenko and Alekseeva 1991; Makarovsky et al. 2007). Following Br_2 exposure, changes in the complete blood count in animal models were reflected by a significant increase in blood neutrophil content, neutrophil-to-lymphocyte ratio, platelet concentration, mean platelet volume, platelet-to-lymphocyte ratio, and systemic immune-inflammatory index. These changes suggest an active acute inflammatory process. These are valuable tools to determine a patient's prognosis in clinical settings as they demark an increase in the risk of thrombosis, cardiovascular injury, and acute lung injury, among others. Higher values of the aforementioned tests suggest a worse outcome, making this an essential diagnostic and prognosis tool (I. Zafar et al. 2021). Other markers of inflammation present in Br-induced toxicity models are increased values of LDH, IL-6, and heme in plasma (Aggarwal et al. 2016). Both LDH and IL-6 have also been used as a prognostic tool after a systemic inflammatory response (Yu et al. 2017; Liu et al. 2020). A summary of these markers is provided in Table 3.

Table 3 Systemic biomarkers of Br_2-induced injury. Brain injury can be assessed by GFAP measurements in plasma. Br-FA and Br-FAD are not clinically relevant at this point. They present plenty of promise as a tool to assess the level of exposure. Changes in glycemia, total cholesterol, and bilirubin are altered after bromine exposure. At the same time, inflammatory markers such as erythrocyte sedimentation rate, IL-6, heme, and LDH present valuable tools for assessing the body's response to this toxic insult (Doumas and Wu 1991; Hopp et al. 2020; Ruutiainen et al. 1981)

Biomarkers	Methods	Other clinical applications
GFAP	ELISA	Brain injury, brain tumors, MS
Br-FA	Liquid chromatography/mass spectrometry	N/A
Br-FAD	Liquid chromatography/mass spectrometry	N/A
Glycemia	Glucose analyzer (electrochemical)	Glucose metabolism disorders, diabetes
Total cholesterol	Gas chromatography (GC), liquid chromatography (LC), and mass spectrometry	Hypercholesterolemias, cardiovascular disorders
Bilirubin	enzymatic methods, HPLC, direct diazo reaction	Inherited bilirubin metabolism disorders, anemias, tumors, liver disease
ESR	Westergren method and the Wintrobe Method	Inflammatory disorders
CBC	Hematology analyzer (electrical impedance, flow cytometry, and fluorescent flow cytometry)	Inflammation, anemia, leukemia, thalassemia, sickle cell anemia, other hemoglobin disorders
LDH	Spectrophotometry	Tissue damage
IL-6	IL-6 immunoassay, in-vitro diagnostic test for the quantitative determination of interleukin-6 in human serum and plasma	Inflammation marker
Heme	Absorbance, fluorescence, mass spectroscopy	Inflammatory diseases

Conclusions

Bromine exposure events are rare, and therefore the gap in knowledge on how to approach them is significant. The use of diagnostic tools from other injury models can be helpful in the absence of specific markers of Br_2-induced injury, as these are still in experimental stages. The assessment of end-organ injury and systemic inflammation can still help determine the extent and severity of the injury.

Mini-Dictionary of Terms

- *Acute respiratory distress syndrome:* It is a life-threatening condition characterized by poor oxygenation and pulmonary infiltrates. The pathophysiological mechanism involved is associated with capillary endothelial injury and diffuse alveolar damage (Diamond et al. 2021).
- *Cardiocirculatory collapse*: It is the state in which the cardiovascular system cannot sustain perfusion pressure to the body's organs. It is a common feature of uncontrolled vasodilation and the inability to maintain cardiac function (Kounis et al. 2018).
- *Calpains:* Calcium-dependent proteases that play a crucial role in apoptotic and necrotic processes (Ahmad et al. 2019).
- *IDHL:* Represents the concentration at which a person can escape at 30 min of exposure without any escape impairing symptoms or any irreversible health effects (Makarovsky et al. 2007).
- *Mass exposure event:* Event of chemical exposure that can cause hundreds of casualties. Triage procedures to identify the individuals with the most urgent need for medical intervention are necessary in this case (Goransson Nyberg et al. 2011).
- *Neutrophil-to-lymphocyte ratio:* An increase in the number of neutrophils in relation to the number of lymphocytes indicates poor prognosis in cases of systemic inflammation. It is of particular use in respiratory distress syndrome (Wang et al. 2018).
- *Platelet-to-lymphocyte ratio:* A novel inflammatory marker, which may be used in many diseases for predicting inflammation and mortality. It can be easily calculated and is widely available (Balta and Ozturk 2015).
- *Systemic immune-inflammatory index:* Value that is measured using the following formula: platelets x neutrophils/lymphocytes. It is used as a prognosis tool in cases of systemic inflammation (Li et al. 2018).

Key Facts on Bromine

- Br_2 toxicity is higher than chlorine and ammonia, as shown by IDLH values for 3 ppm, 10 ppm, and 300 ppm.
- Br_2 has a pungent odor. The odor threshold is 0.05 ppm to 3.5 ppm.
- As a liquid, Br_2 presents with brown color. Once it volatilizes, it turns into an orange gas.

- Br_2 at concentration 1 ppm causes mild irritat, at 10 ppm it causes severe irritation, at 40–60 ppm, it becomes dangerous to the health with toxic pneumonitis and pulmonary edema, and at 1000 ppm it becomes lethal within a few minutes.
- Duration of exposure and concentration are the main determinants of the extent of the injury.
- Br_2 is water-soluble at concentrations of 4%.
- Br_2 is a powerful oxidizing agent, especially in the presence of water.
- Br_2 is not combustible but may cause fire when in contact with combustibles.
- Br_2 is capable of dissolving metals and nonmetals. However, it will not corrode platinum, lead, or nickel.
- It is estimated that 20,000 workers in the USA are at potential risk of occupational bromine exposure. (O.M.J. Kasilo 1999; Makarovsky et al. 2007).

Summary Points

- The Br_2 exposure to tissues on the human body results in oxidative injury to the cells it comes in contact with, causing inflammation and chemical burns.
- The extent and severity of the injury are determined by the concentration of Br_2 and the duration of the exposure.
- Br_2 leaks present the risk for mass exposure events, making the availability of biomarkers to assess the condition of patients of paramount importance.
- Upon entry to the airways, Br_2 causes acute respiratory distress syndrome while also reacting with epithelial cells and generates brominated reactants that carry bromine through the circulation to various organs in the body.
- The impact of Br_2 on the cardiovascular system mimics that of ischemia-reperfusion injury in its mechanism.
- Physiological parameters are good indicators of the body's ability to sustain function after exposure. A worsening of these suggests a poor prognosis.
- Molecular markers of cellular injury, inflammation, and oxidative stress can provide a tool for accurately assessing the severity of the injury the body suffers after Br_2 exposure.
- Br-FA and Br-FAD have shown a correlation with other biomarkers of injury, both physiological and molecular, and therefore should be explored to determine the viability of future clinical applications (Juncos et al. 2020).

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Biomarkers of $PM_{2.5}$ Exposure: Use of Metabolomics as a Platform

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Contents

Abstract

Pollution of particulate matter having an aerodynamic diameter of 2.5 μm or less ($PM_{2.5}$) is the most rapidly increasing cause of global burden of diseases. Biomarkers are frequently assessed to reflect the level of personal exposure to environmental stressor. Identification of biomarkers for $PM_{2.5}$ exposure, therefore, may greatly facilitate the research on both epidemiology and toxicology of $PM_{2.5}$ exposure. The massive methodological advance in untargeted metabolomics has made it possible to unbiasedly analyze the metabolome and then identify potential biomarkers for $PM_{2.5}$ exposure. Therefore, in the past years, there is a continued scientific effort to search biomarkers for $PM_{2.5}$ exposure. This chapter goes on to review these untargeted metabolomic studies and discuss

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potential biomarkers identified by these studies as well as remaining work needed to establish biomarkers for $PM_{2.5}$ exposure.

Keywords

$PM_{2.5}$ · Metabolome · Untargeted metabolomics · Biomarker · Lactate · Citrate

Abbreviations

CAP	Concentrated ambient $PM_{2.5}$
CE	Capillary electrophoresis
COX2	Cyclooxygenase 2
GC	Gas chromatography
LC	Liquid chromatography
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
PGI_2	Prostacyclin I_2
$PM_{2.5}$	Particulate matter that are 2.5 microns or less in aerodynamic diameter
SCFA	Short chain fatty acid
TCA	Tricarboxylic acid

Introduction

Ambient pollution of particulate matter that are 2.5 microns or less in aerodynamic diameter ($PM_{2.5}$, or fine particulate matter) is one of the leading challenges for the global public health (Tong 2019; Bowe et al. 2019; Bu et al. 2021). It was estimated that over 92% of the world's population are living in areas where the pollution level is above the guideline of the World Health Organization (WHO 2020). In addition, considerable studies have shown that all exposure to $PM_{2.5}$ is harmful, regardless of how low the dose is (EPA 2020). In line with these facts, $PM_{2.5}$ pollution now is the most quickly increasing disease burden around the world (Cohen et al. 2017), and thus, there is obviously an urgent need of sensitive and reliable methods to assess the $PM_{2.5}$ exposure level of humans or animal models, which are essential for not only assessing the various health effects due to $PM_{2.5}$ exposure but also delineating the biological mechanism as to how $PM_{2.5}$ exposure provokes these health effects. It is well known that, in addition to natural sources (e.g., soil, dust, sea salt, bioaerosols), anthropogenic sources, including both primary sources (e.g., incomplete combustion, automobile emissions, dust, cooking) and secondary sources (e.g., chemical reactions in the atmosphere), may largely contribute to the local $PM_{2.5}$ pollution too (Liang et al. 2016). Given the multi-sources of $PM_{2.5}$, it is unsurprising that there are huge temporospatial variations in both the concentrations and chemical compositions of $PM_{2.5}$. Although systems were developed, making precise assessment of personal $PM_{2.5}$ exposure possible, the high cost of high-quality personal $PM_{2.5}$ exposure monitors and their complicatedness to use markedly hinders their extensive

application in research (Williams et al. 2014). In contrast, increasing studies have shown that a remarkable variation in the concentration and/or chemical composition of $PM_{2.5}$ may be noted even in a small area (EPA 2020), raising a serious concern over the assessment of $PM_{2.5}$ exposure using immobile equipment, especially in human studies. All these clearly justify the urgent need of research efforts to find out alternatives to assessment of $PM_{2.5}$ concentration and/or composition.

A biomarker refers to a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention (BEST 2016), and thus, measuring a biomarker can be used as an alternative to assessing environmental exposure. As per the measurement method, biomarkers can generally be grouped into four types: molecular, histologic, radiographic, and physiologic (BEST 2016). By far, rapidly increasing studies have demonstrated that $PM_{2.5}$ exposure provokes various histologic, radiographic, and physiologic alterations in humans and animal models (EPA 2020). However, due to difficulty in measurement and/or lack of specificity, these alterations can hardly be used as biomarkers for $PM_{2.5}$ exposure in large-scale study. As a result, in the past decade, especially since the marked methodological advance in untargeted metabolomics has been achieved, there is a continued research effort searching molecular biomarkers for $PM_{2.5}$ exposure using untargeted metabolomics. However, these valuable studies have hardly been reviewed. As such, there is a clear need of summarizing the achievements of this valuable continued research effort and meanwhile identifying remaining technical and conceptional questions in our searching molecular biomarkers for $PM_{2.5}$ exposure.

Untargeted Metabolomics

Metabolomics refers to a large-scale analysis of small molecules (metabolites) in biological samples (e.g., blood, urine, tissue) using high-throughput screening methods and includes targeted metabolomics and untargeted metabolomics. If there is evidence supporting that a group of metabolites are likely influenced by an aimed biological process, a targeted metabolomic analysis can be performed to analyze this group of metabolites only. In other words, targeted metabolomics is used to verify a hypothesis. As such, targeted metabolomics cannot be used to identify new biomarkers. In contrast, untargeted metabolomics is a comprehensive analysis of the total metabolites (metabolome) and thus can be used to identify new biomarkers. Therefore, this review is focused on untargeted metabolomic studies.

By far, almost all the published untargeted metabolomic studies used either mass spectrometry (MS) or nuclear magnetic resonance (NMR) to detect metabolites, albeit an integrated MS/NMR system was most recently developed and shown to have a higher-throughput metabolite annotation (Bhatia et al. 2019). Prior to the detection by MS, individual metabolites need to be separated using liquid chromatography (LC), gas chromatography (GC), and/or capillary electrophoresis (CE). In contrast, such a separation is not required for the detection by NMR. The respective

advantages and disadvantages of MS- and NMR-based metabolomics were systemically reviewed by Dr. Wishart (2019). In brief, although NMR is the analytical technique used in the first metabolomic study about 50 years ago and massive technological advance in NMR-based metabolomics has been made over the past few decades, the more rapid technological developments in MS over the past 10–15 years have made MS-based metabolomics apparently exceed the capability of NMR-based metabolomics. However, given their respective advantages and disadvantages, MS-based metabolomics may not be able to completely replace NMR-based metabolomics, whereas an integrated MS/NMR system is believed to be necessary to further advance the sensitivity, specificity, and accuracy of untargeted metabolomics (Wishart 2019). In addition, the most important practical (e.g., different sample extraction approaches, data acquisition and analysis approaches) and conceptual (e.g., interpretation and extrapolation of results) steps to consider for metabolomic analysis were recently systemically reviewed too and interested readers may be referred to these reviews for details (Defossez et al. 2021; Khodadadi and Pourfarzam 2020; Audano et al. 2021).

Retrieving Relevant Research Studies

One hundred sixty-four published studies in total were retrieved from PubMed using the keywords "particulate matter metabolomics" or "particulate matter metabolome." Forty-five relevant studies were then selected through screening the title and abstract of each retrieved study. The subject (e.g., human, animal, or cultured cells), metabolomic analysis method, particulate matter exposure assessment, and availability of the identified differential metabolites of each relevant study were determined by reading the full text. The inclusion criteria are as follows:

1. In vivo study (the subject is either human or animal model. Studies on cultured cells were not included because it is well known that multiple types of cells in the lung collectively determine the response to inhalation of $PM_{2.5}$ and that $PM_{2.5}$ exposure provokes various systemic health effects through unclear mechanisms (EPA 2020). Therefore, it is really questionable to what extent these cultured cell models can reflect the pathophysiology induced by the real-world $PM_{2.5}$ exposure.
2. Untargeted metabolomic analysis was performed.
3. Particulate matter exposure level was determined (smoking studies were excluded, in consideration of that smoking has potent toxic components other than particulate matter such as nicotine).
4. The list of identified differential metabolites was available.
5. The metabolomic analysis was performed on the blood or urine, in consideration of that $PM_{2.5}$ exposure is well known to have various systemic health effects and the blood and urine are the most frequently used biological samples that can reflect the systemic health effect.

Studies of Human Blood

Given that almost all the interorganal transporting of nutrients and wastes is completed through the blood, it is undoubtedly the best biological sample to search the biomarkers for $PM_{2.5}$ exposure. By far, 16 published studies have investigated the effect of particulate matter pollution on the human blood metabolome using untargeted metabolomics, and 6 of them provided a list of circulating metabolites that were significantly associated with the $PM_{2.5}$ exposure level (Table 1). All these six metabolomic studies used the LC-MS method to determine the effect of $PM_{2.5}$ exposure on human blood metabolome. This remarkable consistency in metabolomic analysis methods largely facilitates comparing and analyzing the results of these six metabolomic studies. Notably, one of them additionally used the GC-MS method (Li et al. 2017) and showed that compared to the LC-MS method, the GC-MS method identified fewer blood metabolites as well as fewer differential blood metabolites due to $PM_{2.5}$ exposure, suggesting that LC-MS is a more sensitive method for searching the biomarkers of $PM_{2.5}$ exposure. However, this study also showed that LC-MS and GC-MS indeed identified distinct blood metabolites and differential blood metabolites due to $PM_{2.5}$ exposure, strongly supporting the notion that none of the current metabolomic analytical techniques can fully replace others and an integration of these techniques is required to have a higher-throughput metabolite annotation (Wishart 2019).

In addition, all these six metabolomic studies collected blood samples in at most 16 h after exposure to $PM_{2.5}$. Therefore, they investigated just the acute metabolomic effect of $PM_{2.5}$ exposure, and thus, the chronic metabolomic effect of $PM_{2.5}$ exposure remains to be determined, given that $PM_{2.5}$ exposure is well known to have chronic health effects, e.g., lung cancer (Raaschou-Nielsen et al. 2013). Of these metabolomic studies, only one study investigated the time lag (of 2 and 16 h) effect of $PM_{2.5}$ exposure on human blood metabolome; the other five studies all collected blood samples immediately after the designed particulate matter exposure (Table 1). In contrast to the high consistency in metabolomic analysis method in these studies, the interventions and thus the designed $PM_{2.5}$ exposures in these studies are remarkably different. One study reduced the personal $PM_{2.5}$ exposure level using air purifiers during a designed duration (Li et al. 2017), and other studies just assessed the average personal $PM_{2.5}$ exposure level of a designed duration. Furthermore, the designed $PM_{2.5}$ exposure durations in these studies are also markedly different (Table 1), varying from the shortest 2-h exposure to the longest 1-year exposure, largely adding to the difference in the designed $PM_{2.5}$ exposures of these metabolomic studies. However, along with their consistency in metabolomic analysis method, this marked variation of these metabolomic studies in the designed $PM_{2.5}$ exposure durations indeed provides an unexpected opportunity to investigate the exposure duration dependence of metabolomic effect of $PM_{2.5}$ exposure. It is noteworthy that while a 2-h exposure to $PM_{2.5}$ was shown not to provoke any significant effect on the blood metabolites (Ladva et al. 2018), all the other longer exposures to $PM_{2.5}$ were found to be significantly associated with at least several

Table 1 Untargeted metabolomic studies of human blood. ↓ decrease versus control; ↑ increase versus control

Reference	Circulation. 136(7):618–627	PLoS One. 13(9): e0203468	Chemosphere. 276: 130102	Environ Res. 201:111553.	Environ Health. 20(1):3
Treatment	36-h removal of $PM_{2.5}$ using air purifier	2-h highway exposure	3-day average of $PM_{2.5}$ exposure	30-day average of $PM_{2.5}$ exposure	365-day average of $PM_{2.5}$ exposure
Analytical method	GC-MS and LC-MS	LC-MS	LC-MS	LC-MS	LC-MS
Sample	Blood	Blood	Blood	Blood	Blood
Differential metabolites	Adenine ↑	None	Bilirubin ↓	5-oxoproline ↓	Aspartate ↓
	Thymine ↓		Biliverdin ↓	Cysteinylglycine ↓	Cys-Gly, Oxidized ↓
	Thymidine ↑		2-Octenoylcarnitine ↓	Alpha-hydroxyisocaproate ↓	Cysteinylglycine disulfide* ↓
	Guanosine ↓		N-Heptanoylglycine ↓	1-carboxyethylleucine ↓	Cysteinylglycine ↓
	7-Methylguanine ↑		Thyrotropin-releasing hormone ↑	Isovalerate (i5:0) ↑	Sarcosine ↑
	1-Methyladenosine ↑		Glutathione ↑	Alpha-hydroxyisovalerate ↓	Alpha-hydroxyisocaproate ↓
	Hydroxylamine ↓		Acetylcysteine ↓	Taurine ↓	Taurine ↓
	Lipoamide ↓		LysoPA(13:0/0:0) ↓	Cysteine s-sulfate ↓	Cysteine S-sulfate ↓
	N-Arachidonoylglycine ↑		PA(14:1/14:0) ↓	5-Methylthioadenosine (MTA) ↑	5-Methylthioadenosine (MTA) ↑
	Oleamide ↑		PE(15:0/14:1) ↑	Arginine ↑	Maltose ↓
	Palmitoleoyl ethanolamide ↑		LysoPE(20:4/0:0) ↓	Mannose ↑	3-Phosphoglycerate ↓
	α-CEHC ↓		N-Acetylserotonin ↓	3-Phosphoglycerate ↓	Lactate ↓

	Thromboxane B2 ↑		Indolepyruvate ↓	Lactate ↓	Ribitol ↓
	6-keto-prostaglandin F1a ↑		Melatonin ↓	Ribitol ↓	Heme ↓
	PE(14:0/14:1n5) ↑			Heme ↓	Bilirubin (E,Z Or Z,E)* ↑
	PC(18:2n6/16:0) ↑			Malate ↓	Bilirubin (E,E)* ↑
	PC(18:2n6) ↑			Oleoylcarnitine (C18:1) ↓	Bilirubin (Z,Z) ↑
	LysoPE(16:0) ↑			Adrenoylcarnitine (C22:4)* ↓	Nicotinamide ↓
	LysoPE(18:0) ↑			Arachidonoyl carnitine (C20:4) ↓	Alpha-tocopherol ↑
	LysoPE(18:1n7) ↑			Glycerophosphoglycerol ↓	Malate ↓
	LysoPE(18:2n6)↑			Glycerol ↓	Succinate ↓
	LysoPE (20:1n9) ↑			1-stearoyl-GPS (18:0)* ↓	1-Stearoyl-GPS (18:0)* ↓
	LysoPE (20:2n6) ↑			1-arachidonoyl-GPA (20:4) ↓	1-Arachidonoyl-GPA (20:4) ↓
	LysoPE (20:4n6) ↑			1,2-dipalmitoyl-GPE (16:0/16:0)* ↓	1,2-Dipalmitoyl-GPE (16:0/16:0)* ↓
	LysoPE (22:5n6) ↑			1-palmitoyl-2-oleoyl-GPE (16:0/18:1) ↓	1-Stearoyl-2-Oleoyl-GPS (18:0/18:1) ↓
	LysoPE (22:6n3) ↑			1-stearoyl-2-oleoyl-GPE (18:0/18:1) ↓	Phosphoethanolamine ↓
	LysoPC(14:0) ↑			1-stearoyl-2-oleoyl-GPS (18:0/18:1) ↓	Sphinganine ↓
	LysoPC (15:0) ↑			Sphinganine-1-phosphate ↓	Sphingomyelin (D18:2/18:1)* ↑
	LysoPC (17:0) ↑			Sphingosine 1-phosphate ↓	Sphingosine ↓

(continued)

Table 1 (continued)

Reference	Circulation. 136(7):618–627	PLoS One. 13(9): e0203468	Chemosphere. 276: 130102	Environ Res. 201:111553.	Environ Health. 20(1):3
	LysoPC (16:0) ↑			Inosine 5′-monophosphate (IMP) ↓	Inosine 5′-monophosphate (IMP) ↓
	LysoPC(P-16:0) ↑			Hypoxanthine ↓	Hypoxanthine ↓
	LysoPC(16:1n7) ↑			Adenosine 5′-monophosphate (AMP) ↓	Allantoin ↓
	LysoPC(18:0) ↑			Uracil ↓	Adenosine 5′-monophosphate (AMP) ↓
	LysoPC(P-18:0) ↑			Gamma-glutamylmethionine ↑	Dihydroorotate ↑
	LysoPC(18:1n9) ↑			Gamma-glutamylglycine ↑	Uracil ↓
	LysoPC(18:2n6) ↑			Gamma-glutamylglutamine ↑	Beta-alanine ↓
	LysoPC(18:3n6) ↑			Ergothioneine ↓	Gamma-glutamylmethionine ↑
	LysoPC(20:2n6) ↑			X – 23739 ↑	Gamma-glutamylalanine ↑
	LysoPC(20:3n9) ↑			X – 11632 ↓	Gamma-glutamylglycine ↑
	LysoPC(20:4n6) ↑			X – 25416 ↑	Gamma-glutamylglutamine ↑
	LysoPC(22:5n3) ↑				Gamma-glutamylvaline ↑
	LysoPC(22:6n3) ↑				Gamma-glutamylisoleucine* ↑

	Sphingosine 1-phosphate ↑				Gamma-glutamyl-alpha-lysine ↑
	Phytosphingosine ↓				Gamma-glutamylthreonine ↑
	Capric acid ↑				Iminodiacetate (IDA) ↑
	Caprylic acid ↑				Hepes ↑
	Sebacic acid ↑				Tartronate (hydroxymalonate) ↑
	Undecanedioic acid ↑				Ergothioneine ↓
	cis-4-Decenoic acid ↑				X – 24,970 ↓
	5-Dodecenoic acid ↓				X – 24,307 ↑
	5,8-Tetradecadienoic acid ↑				X – 23,739 ↑
	Linoleic acid ↑				X – 12,104 ↑
	Eicosapentaenoic acid ↑				X – 11,632 ↓
	Eicosenoic acid ↓				X – 13,684 ↓
	1-Palmitoylglycerol ↓				X – 24,306 ↑
	2-Palmitoylglycerol ↓				X – 24,431 ↓
	2-Eicosapentaenoylglycerol ↑				X – 14,904 ↓
	Carnitine ↑				X – 10,458 ↓
	Acetylcarnitine ↑				
	Stearidonylcarnitine ↑				
	Linolenylcarnitine ↑				
	3-Hydroxysuberate ↑				
	3-Hydroxydodecanedioate ↑				
	3-Hydroxytetradecanedioate ↑				
	9-HOTE ↑				
	Acetone ↑				
	Glucose ↑				

(continued)

Table 1 (continued)

Reference	Circulation. 136(7):618–627	PLoS One. 13(9): e0203468	Chemosphere. 276: 130102	Environ Res. 201:111553.	Environ Health. 20(1):3
	Glucose 6-phosphate ↑				
	Arginine ↑				
	Leucine ↑				
	Histamine ↑				
	Threonine ↑				
	Serine ↑				
	Glutamate ↑				
	Lysine ↑				
	Phenylpyruvate ↑				
	Phenyllactate ↑				
	Phenylethylamine ↑				
	Hydroxyphenyllactate ↑				
	Tyramine ↑				
	Indolelactate ↑				
	Guanidinoacetate ↑				

	4-Guanidinobutanoate ↑				
	Alpha-ketoisovalerate ↑				
	Hydroxyisocaproate ↑				
	N6-Acetyl-L-lysine ↑				
	Cortisol ↑				
	Cortisone ↑				
	Epinephrine ↑				
	Norepinephrine ↑				
	Phenylalanine ↑				
	Tyrosine ↑				
	Tetrahydropteridine ↑				
	L-Tryptophan ↑				
	Serotonin ↓				
	N-Acetylserotonin ↑				
	Melatonin ↑				

blood metabolites (Table 1). These studies collectively showed that the longer the exposure duration was, the more blood metabolites significantly associated with the $PM_{2.5}$ exposure level were found (Table 1). Moreover, Nassan et al. indeed specifically analyzed the association between the circulating metabolites and the average $PM_{2.5}$ exposure levels of preceding 1 day, 7 days, and 30 days. They demonstrated that the longer the analyzed exposure duration was, the more circulating metabolites were found to be significantly associated with $PM_{2.5}$ exposure (Nassan et al. 2021a). All of these strongly suggest a cumulative action mode of $PM_{2.5}$ exposure on the human metabolome.

Given the critical role of exposure duration in the development of adverse health effects, exposures to pollutants are usually grouped into acute ($\leq$14 days), sub-chronic (>14 days and $\leq$ 10% lifespan), and chronic (>10% lifespan) exposures as per their exposure durations (Duffus et al. 2009). The abovementioned cumulative action mode of $PM_{2.5}$ exposure on the human blood metabolome strongly suggests that searching the biomarkers of chronic $PM_{2.5}$ exposure may be of more public health significance and also highly likely an easier job than searching the biomarkers for acute $PM_{2.5}$ exposure. However, by far, there is only one published study investigating the effect of chronic $PM_{2.5}$ exposure on the blood metabolome using untargeted metabolomics (Nassan et al. 2021b). In this study, Nassan et al. showed that 58 blood metabolites were significantly associated with the annual mean $PM_{2.5}$ exposure level. The pathway enrichment analysis using these $PM_{2.5}$ exposure-associated blood metabolites revealed that chronic exposure to $PM_{2.5}$ was significantly associated with the glycerophospholipid, propanoate, sphingolipid, and glutathione metabolic pathways. Notably, Nassan et al. also investigated the effect of sub-chronic $PM_{2.5}$ exposure on the blood metabolome using untargeted metabolomics (Nassan et al. 2021a). They found that the 30-day mean $PM_{2.5}$ exposure level was significantly associated with 40 blood metabolites as well as the sphingolipid and beta-alanine metabolic pathway. By comparing these two lists of $PM_{2.5}$ exposure-associated blood metabolites, we found 23 $PM_{2.5}$ exposure-associated metabolites shared by both studies, clearly demonstrating that sub-chronic and chronic $PM_{2.5}$ exposures are remarkably similar in their effects on the human blood metabolome. To explore the metabolic pathways influenced by both sub-chronic and chronic $PM_{2.5}$ exposures, we performed the metabolic pathway enrichment analysis (MetaboAnalyst 5.0) (Pang et al. 2021) using these 23 shared $PM_{2.5}$ exposure-associated metabolites and found that the glycolysis/gluconeogenesis pathway was the sole significantly enriched metabolic pathway. This is obviously consistent with rapidly increasing published studies showing the association between $PM_{2.5}$ exposure and the abnormalities in glucose homeostasis (Alemayehu et al. 2020; Bailey et al. 2020; Li et al. 2019). However, by far, none of these 23 significant associations between human circulating metabolites and $PM_{2.5}$ exposure levels was yet confirmed by other untargeted or targeted analyses. Therefore, further study is needed to determine whether these blood metabolites can be used as reliable biomarkers for long-term $PM_{2.5}$ exposure.

In direct contrast to the remarkably consistent effects of sub-chronic and chronic $PM_{2.5}$ exposures on the human blood metabolome, the effect of acute $PM_{2.5}$

exposure on the human blood metabolome appears to be distinct. It is noteworthy that the published studies investigating the blood metabolomic effect of acute $PM_{2.5}$ exposure not only showed distinct blood metabolomic effect of acute $PM_{2.5}$ exposures from that of chronic $PM_{2.5}$ exposures but also showed that the blood metabolomic effects of acute $PM_{2.5}$ exposure were almost completely different from each other. For example, while most studies investigating the effect of $PM_{2.5}$ exposure on the human blood metabolome are cohort studies, Li et al. performed a randomized controlled trial study to determine the effect of acute $PM_{2.5}$ exposure on human blood metabolome (Li et al. 2017). In this study, air purifiers had been used to decrease indoor $PM_{2.5}$ for 36 h. It has been shown that this decrease in $PM_{2.5}$ exposure using air purifier significantly altered the levels of 97 blood metabolites. Most of these differential metabolites are related to the metabolism of glucose, amino acids, and lipids. Intriguingly, the reduction of $PM_{2.5}$ exposure with air purifiers was also found to significantly decrease a considerable number of metabolites that are related to the stress response, suggesting that the stress hormones may be used as biomarkers for $PM_{2.5}$ exposure. This is consistent with several studies showing that $PM_{2.5}$ exposure activated the hypothalamic-pituitary-adrenal axis (Liu et al. 2020a; Ahlers and Weiss 2021; Toledo-Corral et al. 2021; Thomson et al. 2019; Hajat et al. 2019; Thomson 2019; Snow et al. 2018). However, except for an increase in arginine (Li et al. 2017; Nassan et al. 2021a), none of the other alterations in blood metabolites following the reduction of indoor $PM_{2.5}$ level using air purifier was shared by other studies investigating the effect of $PM_{2.5}$ exposure on the human metabolome using untargeted metabolomics. Given the numerous differences among these studies, e.g., different subjects, different $PM_{2.5}$ sources, different metabolomic analysis methods, and different $PM_{2.5}$ exposure durations, considerable further study is obviously needed to address this apparent discrepancy. Arginine is a nonessential amino acid and well known to be the substrate of nitric oxide synthases and thus essential to produce nitric oxide (Ikawa et al. 2020). Notably, supplementation of arginine was most recently shown to mitigate air pollution-associated hypomethylation of platelet mitochondrial DNA, implicating arginine in the pathogenesis due to $PM_{2.5}$ exposure (Liu et al. 2021a). However, a recent targeted study investigating the effect of $PM_{2.5}$ exposure on blood amino acids did not find that $PM_{2.5}$ exposure significantly increased blood arginine (Hu et al. 2021). Therefore, caution still needs to be taken when using the blood arginine level as a biomarker for acute $PM_{2.5}$ exposure.

It is noteworthy that in addition to the abovementioned studies that investigated the immediate blood metabolome effect of $PM_{2.5}$ exposure, one published study assessed the time-lag effects of acute $PM_{2.5}$ exposure on the human metabolome (Vlaanderen et al. 2017). Interestingly, this study showed comparable numbers of human blood metabolites significantly associated with $PM_{2.5}$ exposure 2 or 18 h after a 5-h exposure to $PM_{2.5}$ (Vlaanderen et al. 2017). However, these two lists of $PM_{2.5}$ exposure-associated metabolites did not have any overlap, suggesting that the effect of acute $PM_{2.5}$ exposure on the blood metabolome may be highly dynamic. Given that this study was primarily aimed to investigate associations between blood metabolites and $PM_{2.5}$ components rather than $PM_{2.5}$ in whole and the

pathophysiology induced by different components of $PM_{2.5}$ may indeed have different time courses, it remains to be determined whether the apparently dynamic effects of acute $PM_{2.5}$ exposure on the human blood metabolome reflect the complicated composition of $PM_{2.5}$ or just different stages of a pathogenesis. By far, few studies have ever investigated the time-lag effects of chronic $PM_{2.5}$ exposure on the human metabolome. However, given the abovementioned cumulative action mode for $PM_{2.5}$ exposure impacting the human blood metabolome, it is expected that the blood metabolomic effect of chronic $PM_{2.5}$ exposure may be indolent. Since this indeed suggests that to find out the biomarkers of chronic $PM_{2.5}$ exposure may be relatively easier than to find out the biomarkers of acute $PM_{2.5}$ exposure, further study is urgently needed to determine the time-lag effects of chronic $PM_{2.5}$ exposure to prove this notion.

Studies of Human Urine

Urine is another type of frequently used human samples to search biomarkers for $PM_{2.5}$ exposure. By far, the published untargeted metabolomic studies on human urine investigated the immediate effect of acute $PM_{2.5}$ exposure only (Table 2). Therefore, to obtain appropriate urine biomarkers for $PM_{2.5}$ exposure, further studies are needed not only to determine the time lag effect of $PM_{2.5}$ exposure on urine metabolome but also assess the urine metabolome effect of chronic $PM_{2.5}$ exposure. Notably, the current untargeted metabolomic studies showed that the human urine versus the human blood had remarkably fewer metabolites and fewer $PM_{2.5}$ exposure-associated metabolites, suggesting that the blood may be a more sensitive sample for searching the biomarkers of $PM_{2.5}$ exposure using untargeted metabolomics (Table 2). Consistent with the abovementioned inconsistency in blood metabolomic effects of acute $PM_{2.5}$ exposure, the current published studies investigating the urine metabolomic effect of acute $PM_{2.5}$ exposure did not have any overlap in the metabolites significantly associated with acute $PM_{2.5}$ exposure too. However, it remains to be determined whether this inconsistency reflects the methodological difference of these untargeted metabolomic studies or a remarkable dependence of urine metabolomic effect of acute $PM_{2.5}$ exposure on the composition of $PM_{2.5}$. In addition, it is noteworthy that acute $PM_{2.5}$ exposure-associated increases in glucose (Li et al. 2017; Liu et al. 2020b) and 6-keto-prostaglandin F1a (Li et al. 2017; Chen et al. 2019) were both shared by one human urine study and one human blood study. As mentioned above, $PM_{2.5}$ exposure is well known to be associated with abnormal glucose homeostasis (Alemayehu et al. 2020; Bailey et al. 2020; Li et al. 2019). This increase in urine glucose is consistent with considerable epidemiological studies showing the association between $PM_{2.5}$ exposure and blood glucose level (Alemayehu et al. 2020; Bailey et al. 2020; Li et al. 2019). 6-keto-prostaglandin F1a is the inactive hydrolysis product of prostacyclin I_2 (PGI_2) (Pace-Asciak 1976). It was shown that exposure to diesel exhaust significantly increased the urine 6-keto-prostaglandin F1a level in a mouse model as a result of upregulation of cyclooxygenase

Table 2 Untargeted metabolomic studies of human urine. ↓ decrease versus control; ↑ increase versus control

Reference	Environ Int. 138: 105663	Environ Int. 130:104920	Environ Int. 130:104878	Environ Int. 121 (Pt 2):1243–1252
Treatment	5-day air purifier	4-h respirator	9-day air purifier	22-h average of PM2.5
Analytical method	LC-MS	LC-MS	LC-MS	LC-MS
Sample	Urine	Urine	Urine	Urine
Differential metabolites	D-glucose ↑	Prolyl-arginine ↑	3D,7D,11D-Phytanic acid ↓	Uric acid ↓
	3-Hydroxyvaleric acid ↓	α-Ketoglutaric acid ↑	12-Hydroxydodecanoic acid ↓	Glyceric acid 1,3-biphosphate ↓
		Molybdopterin precursor Z ↓	Linoleyl carnitine ↓	Methyluric acid ↓
		8-Hydroxy-deoxyguanosine ↓	Vaccenyl carnitine ↓	Indolelactic acid ↑
		5-Hydroxylysine ↑	l-Octanoylcarnitine ↑	5-Phosphoribosylamine ↑
		N-acetylglutamine ↑	2-trans,4-cis-decadienoylcarnitine ↑	Dopamine 4-sulfate ↓
		γ-Glutamyl ornithine ↓	3-Hydroxydecanoyl carnitine ↑	4-Pyridoxic acid ↓
		2-Oxovaleric acid ↓	Decanoylcarnitine ↑	
		5′-Phosphoribosyl-N-formylglycinamide ↓	PS(18:0/20:4) ↓	
		Homovanillic acid ↑	PE(18:3/18:3) ↓	
		l-Citrulline ↑	PA(15:0/22:5) ↓	
			PA(22:1/19:2) ↓	
			PA(22:6/18:4) ↓	
			Sphingosine ↓	
			6-Keto-prostaglandin F1a ↑	
			Palmitoylethanolamide ↓	

2 (COX2) (Bai et al. 2012). This study strongly supports that the urine 6-keto-prostaglandin F1a may be an important biomarker for $PM_{2.5}$ exposure.

Studies of Animal Blood

By far, nine untargeted metabolomic studies tried to seek the blood biomarkers of $PM_{2.5}$ exposure in animal models (Table 3). Of them, three studies investigated the blood metabolomic effect of acute $PM_{2.5}$ exposure, and six studies used chronic $PM_{2.5}$ exposure models. Notably, in direct contrast to the abovementioned cumulative action of $PM_{2.5}$ exposure on the human blood metabolome, these animal blood metabolomic studies did not show a marked dependence of the number of $PM_{2.5}$ exposure-associated blood metabolites on the exposure duration (Table 3). The reason for this apparent discrepancy remains to be determined. In addition, of these animal studies, one acute $PM_{2.5}$ exposure study and one chronic $PM_{2.5}$ studies used the ^{1}H-NMR method to determine the blood metabolomic effect of $PM_{2.5}$ exposure; all the others used the LC-MS method. It is noteworthy that the studies using the LC-MS method appeared to identify more $PM_{2.5}$ exposure-associated blood metabolites than those using the ^{1}H-NMR method, supporting that the LC-MS method is a more sensitive technique for searching the biomarkers of $PM_{2.5}$ exposure (Wishart 2019).

As described above, the sub-chronic and chronic $PM_{2.5}$ exposures were shown to provoke considerably similar effects on the human blood metabolome (Nassan et al. 2021a, b). In contrast, few $PM_{2.5}$ exposure-associated blood metabolites were shared by the current animal model studies that investigated the blood metabolomic effect of chronic $PM_{2.5}$ exposure using untargeted metabolomics. It is noteworthy that the studies investigating the human blood metabolomic effect of sub-chronic or chronic $PM_{2.5}$ exposure were performed by the same research group, whereas the animal model studies were from different research groups, suggesting that the inconsistency of blood metabolomic effects of chronic $PM_{2.5}$ exposures in animal models may be due to the methodological differences among these studies. Though no significant association between $PM_{2.5}$ exposure and animal blood metabolite was shared by the majority of untargeted metabolomic studies, several $PM_{2.5}$ exposure-associated blood metabolites were indeed identified by two or more studies. The most frequently shared alteration in the animal blood metabolome following $PM_{2.5}$ exposure is the decrease in circulating lactate. Two acute $PM_{2.5}$ exposure animal studies (Zhang et al. 2018; Brower et al. 2016) and two chronic $PM_{2.5}$ exposure animal studies (Zhang et al. 2017; Geng et al. 2021) showed that $PM_{2.5}$ exposure was significantly associated with a decrease in the blood lactate level. Notably, a decrease in blood lactate was also shown to be significantly associated with human sub-chronic and chronic $PM_{2.5}$ exposures (Nassan et al. 2021a). Taken together, these published untargeted metabolomic studies strongly suggest that the blood lactate level may be a reliable biomarker for $PM_{2.5}$ exposure. Interestingly, lactate was shown to be one of the most abundant water-soluble organic acids in $PM_{2.5}$

Table 3 Untargeted metabolomic of animal blood. ↓ decrease versus control; ↑ increase versus control; * decrease or increase versus control is dependent on exposure level

Reference	Anal Biochem. 631:114310	Am J Physiol Heart Circ Physiol. 320(5):H1836-H1850	Environ Pollut. 272:115922.	Sci Total Environ. 732:139304	Environ Pollut. 247:953-963	Oncotarget. 9(56):30748-30760	Chemosphere. 207:337-346	Sci Total Environ. 589:212-221	Inhal Toxicol. 28(5):241-50
Animal model	Rats	Mice	Rats	Mice	Mice	Mice	Mice	Rats	Mice
PM2.5 exposure	30-day instillation of PM2.5	9-day CAP exposure	28-day instillation of PM2.5	4-week instillation of PM2.5	10-month CAP	28-day CAP	One instillation of PM2.5	30-day instillation of PM2.5	six-hour inhalation of diesel engine exhausts
Analytical method	LC-MS	LC-MS	LC-MS	LC-MS	LC-MS and GC-MS	LC-MS	NMR	NMR	LC-MS and GC-MS
Sample	Blood	Blood	Blood	Blood	Blood	Blood	Blood	Blood	Blood
Differential metabolites	Oleic acid ↓	Hydroxyproline	Deoxyinosine ↓	Citric acid ↓	1,5-Anhydroglucitol ↓	Benzo(a) pyrene ↑	HDL ↑	Lactate ↓	sarcosine (N-Methylglycine) ↓
	2,4,12-Octadecatrienoic acid isobutylamide ↓	1-Stearoyl-2-arachidonoyl-GPC 18:0/20:4	LPC 18:0 ↓	L-Arginine ↓	Melatonin ↓	Niacinamide ↑	VLDL ↓	Alanine ↓	glutamate ↑
	Arachidic acid ↓	1-Stearoyl-2-oleoyl-GPC 18:0/18:1	Androsterone sulfate *	L-Tyrosine ↓	Tagatose ↓	Phenanthrene ↑	Isoleucine ↑	Dimethylglycine ↓	histidine ↓
	LysoPC(0:0/16:0) ↓	1-stearoyl-2-arachidonoyl-GPE 18:0/20:4	LPC 20:4 ↓	Phenylacetylglycine ↑	D-Arabitol ↑	Acenaphthene ↑	Leucine ↑	Creatine ↓	phenylacetylglycine ↓
	LysoPC(18:1(9Z)) ↓	Homocitrulline	orotidine-5-phosphate ↑		4-hydroxyphenylacetic acid ↑	Citrate ↑	Valine ↑	Glycine ↓	isovalerylcarnitine ↓
	LysoPE(20:0/0:0) ↓	N,N-dimethylalanine	LPC 20:5 ↓		Uracil ↑		Lipid ↓	histidine ↓	2-methylbutyrylglycine ↑
	10,20-Dihydroxyeicosanoic acid ↑	Glycerophosphorylcholine (GPC)	S1P *		O-Phosphoserine ↓		Lactate ↓		isobutyrylglycine ↓
	PC(22:2(13Z,16Z)/14:0) ↓	2-Stearoyl-GPE 18:0	L-threonine *		1,2,4-Benzenetriol ↑		Lysine ↑		5-methylcysteine ↑
	Nutriacholic acid ↓	Glucuronate	Glutamate *		Diglycerol ↓		Glutamine ↑		ornithine ↓
	Sphinganine ↓	Fructosyllysine	LPC 18:3 ↓		Butyraldehyde ↓		methylguanidine ↓		5-oxoproline ↑
		N-acetylserine	SM(d18:2/22:0) ↑		D-alanyl-D-alanine ↓		Creatine ↑		gamma-glutamyltryptophan ↑
		1-Stearoyl-GPE 18:0	PC(38:6) ↑		2,3-Dihydroxypyridine ↑		α-Glucose ↑		bradykinin, des-arg(9) ↑
		Threonate	PC(32:0) ↑		Behenic acid ↑		Threonine ↑		TDTEDKGEFLSEGGGVR ↑
		1-Stearoyl-2-linoleoyl-GPE 18:0/18:2	FFA 20:1 *		Prostaglandin A2 ↓		Triglycerides ↓		1,5-anhydroglucitol (1,5-AG) ↓
		S-methylmethionine	PC(39:1) ↑		2-ketobutyric acid ↓		Histidine ↑		lactate ↓
		3-Indoleglyoxylic acid	D-gluconate ↓		L-Malic acid ↑		Phenylalanine ↑		ribose ↓
		Trimethylamine N-oxide	PC(40:5) ↑		Turanose ↑				raffinose ↓
		Cholesterol	Choline glycerophosphate ↓		Lyxose ↓				mannitol ↑
		1-Stearoyl-2-docosahexaenoyl-GPC 18:0/22:6	FFA 20:2 *		Threonic acid ↑				alpha-ketoglutarate ↑
		Palmitoleate 16:1n7	Spermidine *		Oxoproline ↑				pyrophosphate ↓
		Glycerophosphoglycerol	Carnitine ↓		Tyramine ↑				caproate (6:0) ↑
		1-Linoleoyl-2-arachidonoyl-GPC 18:2/20:4n6	PC(34:1) ↑		Tricetin ↓				palmitate (16:0) ↓
		Fructose	Cystine ↑		Erythrose 4-phosphate ↓				stearate (18:0) ↓
		1-Oleoyl-2-linoleoyl-GPE 18:1/18:2	PC(36:2) ↑		Fructose ↑				nonadecanoate (19:0) ↓
		Gulonate	5-oxoproline ↓		Octadecanol ↑				arachidate (20:0) ↓
		1-1-Enyl-stearoyl-2-arachidonoyl-GPE P-18:0/20:4	MG 18:1 ↓		Methyl Palmitoleate ↑				erucate (22:1n9) ↓
		1,2-Dilinoleoyl-GPC 18:2/18:2	MG 16:0 ↓		Phenylacetic acid ↓				eicosapentaenoate (EPA; 20:5n3) ↓
		Cystathionine	PC(38:4) ↑		Creatine ↑				dihomo-linolenate (20:3n3 or n6) ↓
		9,10-DiHOME	LPC 18:2 ↓		Beta-Guanidinopropionic acid ↑				docosadienoate (22:2n6) ↓
		Heptadecanedioate C17-DC	PC(38:7) ↑		2E,4E-undecadienoic acid ↑				hexadecanedioate ↑
		Indole-3-carboxylate	SM(d18:2/24:1)		5-Methoxytryptophol ↑				octadecanedioate ↑
		Hydroxyasparagine	LPC 18:1 *		Neurine ↓				valerylcarnitine ↓
		N-acetylcitrulline	LPC O-16:0 ↓		Adrenochrome o-semiquinone ↓				scyllo-inositol ↓

(continued)

Table 3 (continued)

		5-Hydroxyhexanoate	FFA 10:0 *		12-oxo-5E,8E,10Z-dodecatrienoic acid ↑			1-lignoceroylglycerophosphocholine (24:0) ↓
		N-acetylhomocitrulline	FFA 20:3 *		5a-Tetrahydrocortisol ↑			1-palmitoylglycerophosphoethanolamine ↓
		Arachidoylcarnitine C20	PC(40:6) ↑		Iso-Valeraldehyde ↑			2-palmitoylglycerophosphoethanolamine ↓
		1-Palmitoyl-2-linoleoyl-GPE 16:0/18:2	PC(33:1) ↑		4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol ↑			1-linoleoylglycerophosphoethanolamine ↓
		1-Ribosyl-imidazoleacetate	SM21:0 ↑		PI(P-16:0/12:0) ↑			2-linoleoylglycerophosphoethanolamine ↓
		Mannonate	Phenylpyruvate *		Citric acid ↑			1-arachidonoylglycerophosphoethanolamine ↓
		Biliverdin	PC(36:6) ↑		Farnesol ↑			2-arachidonoylglycerophosphoethanolamine ↓
		Galactonate	LPC 16:0 ↓		Formyl 2E,4E,6Z-decatrienoate ↑			1-eicosatrienoylglycerophosphoethanolamine ↓
		3-Hydroxypalmitate	SM22:0 ↑		Eicosapentaenoic acid ↓			1-docosahexaenoylglycerophosphoethanolamine ↓
		Oleate/vaccenate 18:1	PC(36:0) ↑		PA(P-16:0/15:1(9Z)) ↑			1-palmitoylglycerophosphoglycerol ↑
		Myristate 140	PC(33:2) ↑		PE(P-16:0/0:0) ↑			1-stearoylglycerol (1-monostearin) ↑
		1-Stearoyl-2-oleoyl-GPG 18:0/18:1	Methylstearate *		PE(22:4(7Z,10Z,13Z,16Z)/P-18:1(11Z)) ↓			1-oleoylglycerol (1-monoolein) ↓
		13-HODE or 9-HODE	PEA *		L-Alanine ↑			1-linoleoylglycerol (1-monolinolein) ↓
		12,13-DiHOME	PC(31:0) ↑		16,17-epoxy-DHA ↑			2-linoleoylglycerol (2-monolinolein) ↓
		1-Stearoyl-2-docosahexa enoyl-GPE 18:0/22:6	Carbamoyl phosphate *		PC(13:0/0:0) ↑			1-arachidonylglycerol ↓
		10-Heptadecenoate 17:1n7	cis-Aconitate *		PC(10:0/4:0) ↓			stearoyl sphingomyelin ↓
		1-Palmitoyl-2-?-linolenoyl-GPC 16:0/18:3n6	2,6 dimethylheptanoyl carnitine *		3-Methylpyrrolo[1,2-a] pyrazine ↑			deoxycholate ↑
		3-Hydroxystearate	Xanthosine *		17-HDoHE ↑			taurodeoxycholate ↑
		2-Oxoadipate	Guanidoacetic acid *		PS(19:1(9Z)/22:4(7Z,10Z,13Z,16Z)) ↓			tauroursodeoxycholate ↑
		10-Nonadecenoate 19:1n9	FFA 15:0 ↓		4,10-undecadiynal ↓			inosine ↓
		Taurocholate	PC(32:1) ↑		1,12E-Pentadecadiene ↑			hypoxanthine ↓
		Taurodeoxycholate	LPC 20:3 ↓		5,6-Epoxy-8,11,14-eicosatrienoic acid ↑			xanthine ↓
		S-3-hydroxypropyl mercapturicacid (HPMA)	LPC 22:6 ↓		LysoPC(20:5(5Z,8Z,11Z,14Z,17Z)) ↓			guanosine ↓
		3-Phenylpropionate (hydrocinnamate)	PC(36:4) ↑		1alpha-hydroxy-23-[3-(1-hydroxy-1-methylethyl)phenyl]-22,22,23,23-tetradehydro-24,25,26,27-tetranorvitamin D3 / 1alpha-hydroxy-23-[3-(1-hydroxy-1-methylethyl)phenyl]-22,22,23,23-tetradehydro-24,25,26, 27-tetranorcholecalciferol ↑			methylphosphate ↑
		3-Hydroxymyristate	PC(34:2) ↑		18-Oxocortisol ↑			4-hydroxyhippurate ↓
		S-3-hydroxybutyrylcarnitine	SM20:0 ↑		1-Decene ↑			Isobar: 1-kestose, levan ↓

(continued)

Table 3 (continued)

		2-Methylbutyrylcarnitine C5	Urate *		PS(16:0/22:2(13Z,16Z)) ↑				stachydrine ↓
		1-Linoleoyl-2-linolenoyl-GPC 18:2/18:3	FFDA C22:2 ↓		5-HEPE ↑				
		Malonate	PC(36:5) ↑		SM(d16:1/18:1) ↑				
		3-Hydroxylaurate	Lactate *		PE(16:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)) ↑				
		Lyxonate	FFA 22:0 ↑		15S-HpEDE ↑				
		5-Hydroxylysine	Aspartate ↓		D-Limonene ↑				
		Linoleate 18:2n6	FFA 18:1 ↑		PE(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0) ↑				
		10-Undecenoate 11:1n1	FFA 20:4 ↑		Stearic acid ↑				
		Dodecadienoate 12:2	FFA 24:0 ↑		Retinal ↑				
		Hexadecadienoate 16:2n6	FFA 18:3 *		PS(17:1(9Z)/0:0) ↑				
		N2,N2-dimethylguanosine	LPC 14:0 ↓		(±)-2-Methylthiazolidine ↑				
		Sphingomyelin d18:2/18:1	Acetyl-CoA *		GlcCer(d18:1/14:0) ↑				
		Linolenate 18:3n3 or 3n6	L-serine ↓		LysoPC(18:3(6Z,9Z,12Z)) ↑				
		Glycerophosphoethanolamine	L-Malic acid *		PE(18:3(6Z,9Z,12Z)/P-18:1(11Z)) ↑				
		1-Stearoyl-GPI 18:0	FFA 16:2 *		PC(16:0/22:5(4Z,7Z,10Z,13Z,16Z)) ↓				
		16-Hydroxypalmitate	Choline ↓		N-heptanoyl-homoserine lactone ↑				
		N-formylmethionine	L-ornithine ↓		11-dehydro-2,3-dinor-TXB2 ↑				
		Carotene diol 1	D-Glucose 6-phosphate ↑		LysoPE(18:0/0:0) ↑				
		Myristoleate 14:1n5	FFA 16:1 *		PS(19:0/0:0) ↑				
		3-Hydroxydecanoate	FFA 22:7 *		PE(O-18:1(1Z)/20:4(5Z,8Z,11Z,14Z)) ↑				
		Hippurate	Asparagine ↓		9S,10S,11R-trihydroxy-12Z,15Z-octadecadienoic acid ↑				
		Eicosenoate 20:1n9 or 1n11	S-methyl-5-thioadenosine ↑		PC(15:0/18:1(11Z)) ↑				
		Cinnamate	Mesterolone ↓		16-fluoro-7Z-hexadecenoic acid ↑				
		Stearidonate 18:4n3	Carnitine C16:0 *		Tauroursodeoxycholic acid ↑				
		2-Hydroxyoctanoate	Dihydrocholesterol ↑		PE(18:0/20:4(5Z,8Z,10E,14Z)(12OH[S])) ↓				
		Phenylpyruvate	FFA 18:2 *		8Z,11Z,14Z-heptadecatrienal ↑				
		Laurylcarnitine C12	isoleucine ↓		N-Ornithyl-L-taurine ↓				
		Hexadecanedioate C16	FFA 22:1 ↑		PC(16:0/P-18:0) ↑				
		Palmitate 160	SM18:0 *		Taurine ↑				
		N-acetyl-ß-alanine	LPE 18:1 ↓		2-chloro-acetic acid ↑				
		Gluconate	Dimethylglycine ↓		1-(alpha-Methyl-4-(2-methylpropyl)benzeneacetate)-beta-D-Glucopyranuronic acid ↑				
		2- or 3-Decenoate 10:1n7 or n8	FFA 17:1 *		3,3-Dibromo-2-n-hexylacrylic acid ↓				
		Palmitoleoylcarnitine C16:1	L-citrulline ↓		Propionic acid ↓				
		Myristoleoylcarnitine C14:1	Guanine *		N-Acetylaminooctanoic acid ↓				
		Formiminoglutamate	LPC 15:0 ↓		LysoPC(18:0) ↓				
		3-Methoxytyrosine	FFA 18:0 ↑		PC(14:0/20:1(11Z)) ↑				
		N-acetylglutamate	FFA 16:0 ↑		3-Methoxytyramine ↑				
			Tyrosine ↓		2E-tetradecenoyl-CoA ↑				
			Pyroglutamic acid ↓		CPA(18:0) ↑				
			Taurine ↓		L-Carnitine ↓				
			Adenine ↓		Coenzymes QH2 ↑				
			Proline ↓		3-hydroxypentadecanoyl carnitine ↑				

(continued)

Table 3 (continued)

			carnitine C5:0 ↓		Taurochenodeoxycholic acid 7-sulfate ↓				
			Methionine ↓		TG(14:1(9Z)/18:1(11Z)/14:1(9Z)) ↑				
			Glyoxylate ↓		Estrone ↓				
			p-Coumaric acid ↓		Leukotriene B4 ↓				
			Lysine ↓		Psychosine sulfate ↑				
			SM(d18:1/18:1) ↑		2-glyceryl-PGD2 ↓				
			LPC O-18:0 ↓		(all-Z)-8,11,14-Heptadecatrienal ↓				
			Oxidized glutathione ↓		9-HODE ↑				
			dTMP ↓		N-(1-Deoxy-1-fructosyl) leucine ↓				
			LPC 16:1 ↓		2-Thiophenecarboxaldehyde ↓				
			a-ketoglutarate ↓		Undecylic acid ↑				
			LysoSM(d18:1) ↓		PE(20:5(5Z,8Z,11Z,14Z,17Z)/0:0) ↑				
			Hippuric acid ↓		PA(P-16:0/20:5(5Z,8Z,11Z,14Z,17Z)) ↓				
					2E-Hexene ↑				
					(-)-Fusicoplagin A ↓				
					PE(22:4(7Z,10Z,13Z,16Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)) ↑				
					PA(18:1(9Z)/22:2(13Z,16Z)) ↑				
					2-hydroxy-nonadecanoic acid ↑				
					MG(0:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0) ↑				
					Maltopentaose ↓				
					Piperidine ↑				
					PC(P-17:0/0:0) ↑				
					Protoporphyrin IX ↓				
					Tryptophanol ↓				
					Glyoxylic acid ↓				
					2-Oxosuccinamate ↑				
					PS(O-18:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)) ↓				
					PS(17:1(9Z)/22:2(13Z,16Z)) ↓				
					PC(18:2(9Z,12Z)/P-18:1(11Z)) ↑				
					PC(15:0/22:4(7Z,10Z,13Z,16Z)) ↑				
					PI(O-18:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)) ↑				
					2-Thiophenethiol ↓				
					PC(22:5(7Z,10Z,13Z,16Z,19Z)/16:0) ↓				
					L-Leucine ↑				
					2,6-Dimethyl-5-hepten-1-ol ↑				
					Triterpenoid ↓				
					Plastoquinol-1 ↑				
					N-palmitoyl phenylalanine ↑				
					Malonylcarnitine ↓				
					1-Phenylethylamine ↓				
					(7'S,8'S)-4,7'-Epoxy-3,8'-bilign-7-ene-3',5-dimethoxy-4',9,9'-triol 4'-glucoside ↑				
					PC(18:1(11Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)) ↓				
					Isopalmitic acid ↑				
					L-Palmitoylcarnitine ↑				
					2R-aminoheptanoic acid ↑				
					Prostaglandin D3 ↓				
					PC(20:3(5Z,8Z,11Z)/P-18:1(11Z)) ↑				

(Tang et al. 2020). However, the decrease in circulating lactate following $PM_{2.5}$ exposure obviously does not directly result from the inhalation of lactate via $PM_{2.5}$ (Phypers and Pierce 2006). Whether the lactate content of $PM_{2.5}$ plays a role (e.g.,

activating a negative feedback loop) in the decrease of circulating lactate following $PM_{2.5}$ exposure remains to be determined.

It is noteworthy that lactate is a short chain fatty acid (SCFA). A rapidly increasing number of studies have demonstrated that the gut microbiota-associated SCFAs can improve gut barrier integrity and glucose and lipid metabolism and regulate the immune system, the inflammatory response, and blood pressure (Wang et al. 2021a). In direct contrast, numerous studies have shown that $PM_{2.5}$ exposure provokes just the opposite effects on glucose and lipid metabolism, the immune system, the inflammatory response, and blood pressure (EPA 2020). Furthermore, several recent studies showed that $PM_{2.5}$ exposure may remarkably impact the composition and/or function of the gut microbiota, including decrease in the abundance of gut SCFA-producing bacteria (Bailey et al. 2020; Wang et al. 2018; Wu et al. 2020; Ran et al. 2021; Liu et al. 2021b, c; Yang et al. 2021; Van Den Brule et al. 2021; Li et al. 2020; Feng et al. 2020). All of these strongly suggest that the decrease in circulating lactate following $PM_{2.5}$ exposure may be subsequent to the decrease in the abundance of gut SCFA-producing bacteria and thus able to explain all these various adverse health effects due to $PM_{2.5}$ exposure. Therefore, there is obviously an urgent need of further study to confirm this potential pathophysiology, which will not only help determine whether the blood lactate level is a reliable biomarker for $PM_{2.5}$ exposure but also will markedly advance our understanding of the pathogenesis due to $PM_{2.5}$ exposure.

Although the fermentation of the gut microbiota is well known to considerably contribute to the circulating lactate pool, the majority of circulating lactate in mammals indeed is the product of glycolysis of mammal cells (Phypers and Pierce 2006). Glycolysis of mammalian cells under anaerobic conditions is long known to produce a large amount of lactate. Furthermore, many types of cultured mammalian cells were shown to produce a large amount of lactate even under aerobic conditions (Phypers and Pierce 2006). All of these lead to the notion that lactate is a metabolic waste product of mammals. However, recent studies in animal models showed that circulating lactate can be the primary source of carbon for the tricarboxylic acid (TCA) cycle and thus of energy in major organs other than the brain (Rabinowitz and Enerback 2020; Hui et al. 2017), resulting in a paradigm shift regarding the physiological role of circulating lactate. The new paradigm believes that the circulating lactate may indeed serve as a universal carbohydrate fuel (Rabinowitz and Enerback 2020). Furthermore, recent studies demonstrated that intravenous injection of a fusion protein that has the activities of both bacterial lactate oxidase and catalase and thus is able to specifically lower the circulating lactate-pyruvate ratio significantly decreased the NADH-NAD^+ ratio in the heart and brain, strongly suggesting that the circulating lactate plays a critical role in tissue redox buffering too (Patgiri et al. 2020). In agreement with these emerging crucial physiological roles of circulating lactate, the concentration of circulating lactate is known to be subjected to tight regulation, primarily through the balance between the whole-body glycolysis level and flux into the TCA cycle and/or neoglucogenesis of the liver and mitochondria-rich tissues such as skeletal and cardiac myocytes and proximal tubule cells (Phypers and Pierce 2006). Therefore, the decrease of circulating lactate

following $PM_{2.5}$ exposure may also result from a misbalance between the whole-body glycolysis level and the flux of lactate into the TCA cycle and/or neoglucogenesis. Notably, considerable studies have shown that $PM_{2.5}$ exposure may lower the consumption of glucose and energy expenditure in both animal models (Rajagopalan et al. 2020; Song et al. 2020; da Silveira et al. 2018; Hu et al. 2017; Liu et al. 2014) and humans (Hwang et al. 2020; Zhan et al. 2021; Bo et al. 2021; Shin et al. 2020; Yu et al. 2020; Riant et al. 2018), suggesting that $PM_{2.5}$ exposure may reduce the whole-body glycolysis level but highly unlikely increase the flux of lactate into the TCA cycle and/or neoglucogenesis. To determine the underlying biological mechanism for the decrease in circulating lactate following $PM_{2.5}$ exposure which will obviously help determine the value of using the blood lactate as a biomarker for $PM_{2.5}$ exposure, further study is urgently needed to determine whether $PM_{2.5}$ exposure decreases circulating lactate through impacting the whole-body glycolysis level but not the flux of lactate into the TCA cycle and/or neoglucogenesis.

Despite being less frequently, decreases in blood carnitine (Geng et al. 2021; Xu et al. 2019), ornithine (Brower et al. 2016; Geng et al. 2021), dimethylglycine (Zhang et al. 2017; Geng et al. 2021), and tyrosine (Geng et al. 2021; Song et al. 2020) as well as increases in leucine (Zhang et al. 2018; Xu et al. 2019) and citrate (Zhang et al. 2017; Li and Lin 2018) following $PM_{2.5}$ exposure were also shared by two or more untargeted metabolomic studies in animal models, warranting further targeted study to test whether these circulating metabolites can be used as biomarkers for $PM_{2.5}$ exposure. In addition, decrease in blood histidine (Brower et al. 2016; Zhang et al. 2017) and increases in blood creatine (Zhang et al. 2018; Xu et al. 2019) following $PM_{2.5}$ exposure were shared by at least two untargeted metabolomic studies too. However, there is at least another untargeted metabolomic study showing the opposite effect of $PM_{2.5}$ exposure on circulating histidine (Zhang et al. 2018) and creatine (Zhang et al. 2017). Therefore, caution should be taken when considering using blood histidine and creatine as biomarkers for $PM_{2.5}$ exposure.

Studies of Animal Urine

By far, three published animal model studies investigated the effect of $PM_{2.5}$ exposure on urine metabolome using an untargeted metabolomic method and also providing a list of $PM_{2.5}$ exposure-associated urine metabolites (Table 4). Of them, one study used the ^{1}H-NMR method (Zhang et al. 2017), and the other two studies used the LC-MS method (Du et al. 2020; Wang et al. 2021b). In line with the abovementioned animal blood metabolomic studies, the LC-MS method appeared to identify more metabolites as well as $PM_{2.5}$ exposure-associated metabolites in the animal urine than the ^{1}H-NMR method (Zhang et al. 2017; Du et al. 2020; Wang et al. 2021b), supporting the abovementioned notion that LC-MS is a more sensitive method for searching the biomarkers of $PM_{2.5}$ exposure. In addition, all these three studies used a chronic $PM_{2.5}$ exposure model: either chronic exposure to concentrated ambient $PM_{2.5}$ (CAP) (Du et al. 2020) or chronic intratracheal instillation of $PM_{2.5}$ solution (Zhang et al. 2017; Wang et al. 2021b). The reason for lacking

Table 4 Untargeted metabolomic studies of animal urine. ↓ decrease versus control; ↑ increase versus control

Reference	Ecotoxicol Environ Saf. 203:111044	Sci Total Environ. 589:212–221	Anal Biochem. 631: 114310
Animal model	Mice	Rats	Rats
PM2.5 exposure	16-week CAP	30-day instillation of PM2.5	30-day instillation of PM2.5
Analytical method	GC-MS and LC-MS	NMR	LC-MS
Sample	Urine	Urine	Urine
Differential metabolites	4-Ethylphenyl sulfate ↑	Allothreonine ↓	2-Indolecarboxylic acid ↑
	Met thr ↑	Lactate ↓	Indole-3-carboxylic acid ↑
	2,6-Xylenol ↑	Alanine ↓	4,6-Dihydroxyquinoline ↑
	Asp trp tyr ↑	Acetate ↓	3-Formyl-6-hydroxyindole ↑
	Thymine-d3 ↑	Succinate ↓	Isoleucyl-proline ↓
	Leu leu val ↑	Citrate ↑	Leucylproline ↓
	N-acetyl-2-carboxy benzenesulfonamide ↓	Trimethylamine ↓	Orciprenaline ↓
	Chenodeoxycholic acid disulfate ↑	Arginine ↑	Methoxamine ↓
	Geranyl monophosphate ↓	Hippurate ↑	Xanthine ↑
	Ala his ala ↑	Allantoin ↑	Oxypurinol ↑
	Ser asn glu ↑	Formate ↓	6,8-Dihydroxypurine ↑
	Duartin ↑		Xanthogalenol ↑
	Latrunculin A ↓		
	Dhap80 ↑		
	Epsilon-caprolactam ↓		
	O-methyldopamine 3-methoxytyramine ↓		
	Nb-trans-feruloylserotonin glucoside ↑		
	Tyr ser glu ↑		
	Tyr cys thr ↓		
	His ala lys ↑		
	Indole-3-carboxilic acid-o-sulfate ↓		
	2-Amino-3-methyl-1-butanol ↑		
	Vanillic acid-4-o-glucuronide ↓		
	Estrone glucuronide ↓		
	Dihydroactindiolide ↑		
	Ribose 1-phosphate ↑		
	Leukotriene E4 methyl ester ↓		
	Asp ala gly ↑		

(continued)

Table 4 (continued)

Reference	Ecotoxicol Environ Saf. 203:111044	Sci Total Environ. 589:212–221	Anal Biochem. 631: 114310
	1-Monopalmitin ↑		
	Pro glu met ↑		
	2,3-Dihydroxypyridine ↑		
	2′-Deoxyadenosine ↑		
	Dibenzofuran ↑		
	Hydantoin, 5-4-hydroxybutyl- ↓		
	Hydroxylamine ↑		
	Uridine ↑		
	Biuret ↓		
	Carbazole ↓		
	Indole-3-acetamide ↓		
	Citric acid ↑		
	Succinic acid ↑		
	Caprylic acid ↑		
	3-Hydroxysuberic acid ↑		
	2,3-Dinor-TXB2 ↑		
	Glutaric acid ↑		
	Glycerol ↑		
	Homovanillic acid ↓		
	D-glycerol 1-phosphate ↓		
	Omega-carboxy-trinor-leukotriene B4 ↓		
	Alpha-ketoisocaproic acid ↓		
	20-oxo-leukotriene B4 ↑		
	10,11-Dihydro-20-trihydroxy-leukotriene B4 ↑		
	6-Hydroxynicotinic acid ↑		
	3, 5-tetradecadiencarnitine ↑		
	Ferulic acid ↑		
	Daidzein ↑		
	3-hydroxy-l-proline ↑		
	P-benzoquinone ↑		
	Fumaric acid ↓		
	Butyrylcarnitine ↓		
	11-Hydroxy-9-tridecenoic acid ↓		
	18-Oxocortisol ↑		
	6-Hydroxymelatonin ↑		
	Adrenaline ↑		
	Prostaglandin E2 ↑		

(continued)

Table 4 (continued)

Reference	Ecotoxicol Environ Saf. 203:111044	Sci Total Environ. 589:212–221	Anal Biochem. 631: 114310
	Aspartic acid ↑		
	N-acetylisatin ↑		
	O-succinylhomoserine ↑		
	Beta-alanine ↑		
	Dihydrofolic acid ↓		
	Alanine ↑		

untargeted urine metabolomic study in acute $PM_{2.5}$ exposure animal models may be primarily due to the consideration that there obviously is an indeterminate time lag between the production and excretion of urine and thus the urine indeed may not be able to reflect the (patho)physiological status at the time when the urine sample is collected.

To search reliable urine biomarkers for chronic $PM_{2.5}$ exposure, we compared all three published lists of chronic $PM_{2.5}$ exposure-associated animal urine metabolites. As a result, we found that an increase in citrate was the sole urine metabolomic effect of chronic $PM_{2.5}$ exposure shared by two or more untargeted animal urine metabolomic studies (Zhang et al. 2017; Du et al. 2020). This is clearly consistent with the abovementioned untargeted animal blood metabolomic studies showing that although the published lists of chronic $PM_{2.5}$ exposure-associated blood metabolites hardly have any overlap, the increase in blood citrate is just one of the few overlaps (Zhang et al. 2017; Li and Lin 2018). Circulating citrate has multiple critical physiological roles, and thus its concentration is known to be finely tuned through tight regulation of production and/or clearance (Costello and Franklin 2016). The renal clearance of citrate is the major factor for the removal of citrate from the blood (Costello and Franklin 2016). As such, the increases of citrate in both the blood and urine following $PM_{2.5}$ exposure suggest that $PM_{2.5}$ exposure may primarily influence the production, but not the clearance, of citrate. This is somehow supported by other targeted studies showing that although exposure to $PM_{2.5}$ was shown to decrease pulmonary expression of citrate synthase (Jin et al. 2019), chronic $PM_{2.5}$ exposure indeed increased the production of citrate by human bronchial epithelial cells (Fu et al. 2019). Taken together, these published studies strongly suggest that the urine and blood levels of citrate may be used as biomarkers for chronic $PM_{2.5}$ exposure. However, further targeted study is still needed to delineate the biological mechanism for this increase in citrate following chronic $PM_{2.5}$ exposure and determine its role in the pathophysiology due to chronic $PM_{2.5}$ exposure.

In addition, in line with the untargeted blood metabolomic studies in animal models, one untargeted urine metabolomic study in animal models showed that $PM_{2.5}$ exposure significantly decreased the urine lactate level too (Zhang et al. 2017). Since considerable studies have demonstrated that the urine lactate level is significantly associated with the blood lactate level (Nikolaidis et al. 2018; Nikolaidis et al. 2017; Hagen et al. 2000), this untargeted urine metabolomic study

strongly supports that $PM_{2.5}$ exposure decreases the circulating lactate and also suggests that the decrease in urine lactate following $PM_{2.5}$ exposure highly likely results from the decrease in blood lactate.

Applications to Prognosis, Other Diseases, or Conditions

In this chapter, the identified biomarkers for $PM_{2.5}$ exposure in published untargeted metabolomic studies have been reviewed. The rapidly increasing untargeted metabolomic studies of human blood collectively showed an apparent dependency of number of $PM_{2.5}$ exposure-associated metabolites on the exposure duration, strongly suggesting a cumulative action mode of $PM_{2.5}$ exposure on human blood metabolome and thus providing a mechanistic insight into the development of adverse health effect following $PM_{2.5}$ exposure from an unprecedented perspective. In addition, although no individual metabolite was ever identified by most untargeted metabolomic studies, several studies both in humans and animal models showed that a decrease in lactate and an increase in citrate were associated with both chronic and acute $PM_{2.5}$ exposures, strongly supporting that lactate and citrate may be reliable biomarkers for $PM_{2.5}$ exposure. If targeted studies can confirm these effects of $PM_{2.5}$ exposure and establish their quantitative relationship with $PM_{2.5}$ exposure, these findings are expected to provide invaluable tools for study on $PM_{2.5}$ exposure, particularly epidemiological study.

Mini-dictionary of Terms (5-15 Terms)

- *Biomarker – any biological characteristic that may represent the (patho)physiological status*
- *Untargeted metabolomics – comprehensive analysis of metabolome using high-throughput analytical method to identify differential metabolites.*
- *$PM_{2.5}$ – particulate matter with an aerodynamic diameter of $\leq$ 2.5 m.*
- *Lactate – a potential biomarker for $PM_{2.5}$ exposure identified by untargeted metabolomics*
- *Citrate – another potential biomarker for $PM_{2.5}$ exposure identified by untargeted metabolomics*

Key Facts of Biomarkers of $PM_{2.5}$ Exposure: Use of Metabolomics as a Platform

$PM_{2.5}$ exposure is one of the leading global public health challenges.

Molecular biomarkers are urgently needed to assess the $PM_{2.5}$ exposure level.

Increasing untargeted metabolomic studies provides potential biomarkers for $PM_{2.5}$ exposure.

Our review of untargeted metabolomic studies reveals that the number of $PM_{2.5}$ exposure-associated blood metabolites depends on the exposure duration, suggesting a cumulative action mode of $PM_{2.5}$ exposure on the blood metabolome.

Lactate and citrate are the most frequently shared metabolites significantly associated with $PM_{2.5}$ exposure.

Summary Points

- *There are rapidly increasing untargeted metabolomic studies investigating the metabolomic effect of $PM_{2.5}$ exposure.*
- *The duration of $PM_{2.5}$ exposure markedly influences its immediate metabolomic effect: the longer the exposure is, the more metabolites are influenced.*
- *The time-lag metabolomic effect of $PM_{2.5}$ exposure has hardly been investigated.*
- *There is a remarkable inconsistency in the immediate metabolomic effects of $PM_{2.5}$ exposure, and the reason for this discrepancy remains to be determined.*
- *Decrease in lactate and increase in citrate are most frequently associated with $PM_{2.5}$ exposures in published untargeted metabolomic studies.*

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Sunscreens: Toxic Effects as Determined by Biomarkers

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Abstract

Biomarkers are known to occur in body fluids such as blood or tissues that can be used as a sign of normal or abnormal processes. Genomic and proteomic approaches have directed the discovery of melanoma-specific biomarkers. Sunscreen that contains UV-filters provides photoprotection to the skin by reflecting, scattering, and absorbing light. Sunscreen can be organic (or chemical) or inorganic (ZnO and TiO_2). Biomarkers may help in the body's attempt to initiate reparative processes. The growth factor erythropoietin (EPO) along with cytokines upregulation are the novel pathways involved during cellular oxidative stress. These are the protective cellular mechanisms against reactive oxygen species. Oxidative stress-based biomarkers are very significant for giving a base to design appropriate interventions to inhibit or alleviate injury. Studies revealed that photosensitized sunscreen ingredients show mitochondrial-induced apoptosis through activation of caspase-3 in human skin keratinocytes (HaCaT) cell line. Sunscreens have also been associated with photoallergic reactions.

Keywords

Biomarkers · Sunscreens · Ultraviolet radiation/UVR · Phototoxicity · UV filters · Contact dermatitis · Photoallergy · Photosensitization · Oxidative stress · Reactive oxygen species/ROS

Abbreviations

8-OHdG	8-Hydroxy-2′-deoxyguanosine
AD	Atopic dermatitis
AVB	Avobenzone
BP	Benzophenone
CCL-1	Chemokine (C-C motif) ligand 1
CXCL 13	C-X-C motif chemokine ligand 13
IFN-γ	Interferon-gamma
IgE antibodies	Immunoglobulin E antibodies
IL-1RA	Interleukin-1 receptor antagonist
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MCF-7 cell line	Michigan Cancer Foundation-7 cell line
NPs	Nanoparticles
OD-PABA	Octyldimethyl-p-aminobenzoic acid
ONOO-	Peroxynitrite
PABA	p-Aminobenzoic acid
PBS	Phosphate-buffered saline

ROS	Reactive oxygen species
TNF-α	Tumor necrosis factor alpha
UVR	Ultraviolet radiation
γ-H2AX	Gamma-H2AX histone variant

Introduction

Ozone depletion has raised the UVB exposure on the surface of the earth. UVB radiation (290–320 nm) is far more potent than UVA radiation to induce skin pigmentation, sunburn, and cell damage. Over the last few decades, due to ozone layer depletion, the frequency of UVB radiation at the surface of the earth has increased. Excess exposure of UVB can be deleterious to human beings by inducing early skin aging and inflammation, cancer, and cell death. UVB produces phototoxic responses either by direct interaction with DNA or via indirect mechanism by generating oxygen free radicals. Many antibiotics drugs, antimalarial drugs (Dwivedi et al. 2012), and anti-inflammatory drugs are involved in skin photosensitization in the process of reactive oxygen species formation. Over the precedent decades, there has been a constant increase in the rate of occurrence of skin cancer. Skin cancer cases are very frequent and can be considered as a public well-being problem. International health authorities suggest people to take some self-protective measures against detrimental effects of UV radiation (UVR) such as avoiding sun exposure during peak hours, using protective clothes, and application of sunscreen in exposed areas. Sunscreen and related enhanced sun protecting lotions or products are great choices to shield the skin against the harmful effects of UVR (Sambandan and Ratner 2011). UV filters used in sunscreen-based products can be organic or inorganic depending on usage of chemical or mineral compounds, respectively. Current sunscreen manufacturers use a mix of numerous inorganic and organic UV filters that provide security against broader spectrum of UV and ultimately providing maximum UV safety (Wissing and Müller 2001). Inorganic filters do not cause skin allergic reactions compared to organic ones (Wong and Orton 2011). However, inorganic ones leave unlikable white marks on skin in the case of elevated quantities of sunscreen products. To circumvent this scenario, market has come up with nanotized form of inorganic UV filters allowing more pleasant sunscreens with transparent and light textures. Nanotechnology has changed the current sunscreen scenario by changing the matter at nanoscale dimensions.

Sunscreen-Induced Skin Toxicity

Many alarming reports have been documented before regarding the toxicity of UV filters including carcinogenic cell damage and disruption of other biological functions. Organic UV filters are normally used in commonly used sunscreens as they can filter UVR with no unpleasant white marks left on the skin. They are more pleasing sunscreen formulations than inorganic ones. Organic filter combinations are

used to attain an adequate sunscreen protection factor (SPF). Though a few photo-unstable organic filters get decomposed under UVR and generate reactive oxygen species (ROS), they are more toxic derivatives and lose their UVR filter properties (Maier et al. 2001). These may be allergenic to trigger photoallergic contact dermatitis. It is a light-mediated skin allergy reaction induced along with some chemical compounds (Bryden et al. 2006).

An association between UV filters (organic) in sunscreen products and photo-allergic contact dermatitis cases has been also reported (Kerr and Ferguson 2010). In previous studies, it was reported that sunscreen ingredients were found to be the chief photo-allergen agents accountable for photoallergic contact dermatitis (Victor et al. 2010). Organic UV filters absorb UVR and change them via chemical reactions leading to their damage and leading to the generation of photo-unstable reactive intermediate, directly in contact with the skin. Reactive intermediate promotes phototoxic or photoallergic reaction and does not provide protection from UV rays-induced damage. Photo-instability is another motive of UV filter due to their ability of ROS generation under or without stimulation of UV light. However, transfer of ROS through the sunscreen is a matter of conflict. Furthermore, the inactivation of organic UV filters by UVR loses their photo-protectiveness (Vanquerp et al. 1999).

Here we have discussed a few UV filters that were used in the formulation of sunscreen such as benzophenone, avobenzone, methylanthranilate, p-aminobenzoic acid (PABA), octocrylene, octyl triazone, salicylate, zinc oxide, and titanium oxide (Fig. 1). Preservatives are largely used important chemicals, in drugs, cosmetics, and hygiene products. Paraben (p-hydroxy benzoic acid) is an ester compound with broad antimicrobial activity and is commonly used as preservatives in sunscreens. These preservatives easily get absorbed in the human skin due to hydrophobic in nature and gets hydrolyzed through esterases (Sung et al. 2012).

Organic Sunscreen Filter Ingredients and Their Toxicity

PABA and their derivatives (e.g., octyldimethyl-PABA (OD-PABA)) trigger photo-allergies. A currently published report addressed the development of photo-contact allergy to PABA and raised the requirement of continued vigilance of these organic UV filters (Waters et al. 2009).

In vitro assay done on PABA has shown that it forms DNA cyclobutene thymine dimers and induces ROS generation under sunlight irradiation in an aqueous solution. DNA cyclobutane thymine dimers are formed after exposure to nonionizing irradiation such as UV rays and are formed when a double liaison is formed between two consecutive thymine bases of DNA. Thymine dimers inhibit DNA polymerase function and that may lead to cell death or cancer if not repaired. It was observed that PABA can generate ROS in PBS (phosphate-buffered saline) solution under sunlight radiation independent conditions (Cantrell et al. 2001). This is very concerning because PBS mimics the extracellular fluid of humans and it might be cytotoxic when present in the extracellular fluid. A recent study had shown that oestrogenic

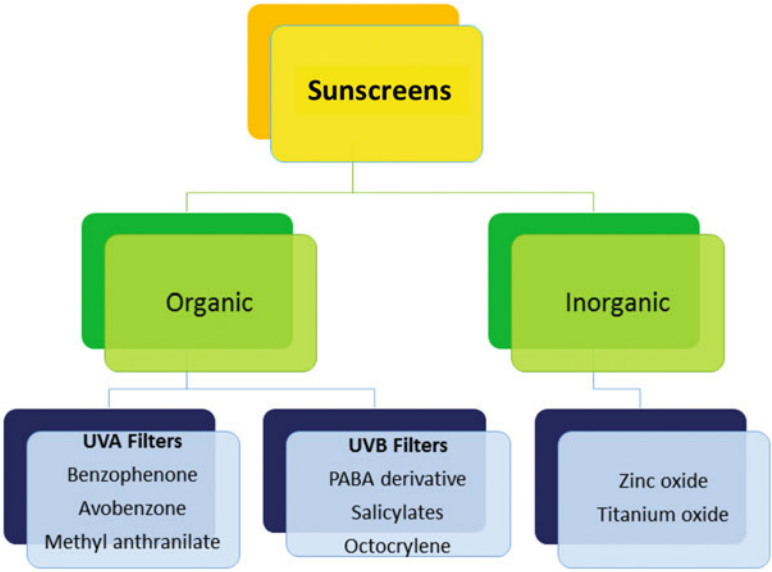

Fig. 1 Types of filters used in sunscreens. Sunscreens are largely divisible into organic and inorganic filters. Organic filters, on the basis of the type of ingredients involved, can give protection in UVA/UVB regions or broad spectrum covering both the regions. Inorganic filters are generally metal oxides that reflect harmful UVR. Along with preservatives, sunscreen gives overall protection against sunlight

activity of OD-PABA on MCF-7 cells leads to their proliferation in vitro (Schlumpf et al. 2004).

Avobenzone (AVB) was declared to be a photoallergic UV filter. It is highly photolabile and photodegrades to aryl glyoxals and benzyls. Aryl glyoxal compounds are potent skin sensitizers. Photo-contact allergy from UV filters could be related to the formation of aryl glyoxals when in contact with the skin. Its photo-instability may cause a 60% decrease in photoprotective properties after sun exposure. Moreover, the stability of other organic UV filters could be affected when they come in contact with AVB. However, these chemical compounds could be stabilized with another organic UV filter, octocrylene, and photo-instability can be avoided. Benzophenone, a commonly used sunscreen ingredient under UVR irradiation, induces oxidative stress, which suppresses the cellular defense and injured the cell organelles (Amar et al. 2018). The concentration of benzophenone in food products ranged from 0.57 to 3.27 μg/ml in nonalcoholic beverages and frozen dairy products. Benzophenone-3 (BP3) penetrates porcine skin when it reaches blood circulation and gets excreted with urine (Kasichayanula et al. 2007). BP3 can reach the systematic blood circulation and can affect other tissues/cells of the organisms. Another study documented oestrogenic activity in three most commonly used benzophenone derivatives (BP1, BP2, and BP3) in organic UV filters. In vitro study has shown that all of the benzophenone derivatives have shown estrogenic

effects on MCF-7 cells (human tumor mammary cells) inducing their proliferation. In vivo study was done on rats using uterotrophic assay to evaluate the oestrogenic activity of BP derivatives. Another study revealed that in in vitro BP3 increases cell proliferation effect on MCF-7 cells, and when an immature Long Evans rat was fed with BP3, a dose-dependent increase in uterine weight was observed.

Octyl triazone is a potent sensitizer which induces photoallergic contact dermatitis in adults and allergic contact dermatitis in children (Avenel-Audran et al. 2010). Salicylate is probably associated with any oestrogenic activity or photoallergic and allergic contact dermatitis and is still incorporated into the sunscreens. In a recent study, it was reported that octyl salicylate leads to the development of allergic contact dermatitis (Gilbert et al. 2013). Octocrylene (OC) can maintain its high UV filter abilities compared to other UV filters (organic) when irradiated. ROS generation by OC in human living epidermal cell layer after being exposed to UV has been well documented.

Inorganic Sunscreen Filter Ingredients and Their Toxicity

The two most common inorganic UV filters namely titanium dioxide (TiO_2) and zinc oxide (ZnO) particles are used in formulations of sunscreen. TiO_2 and ZnO are traditionally used in sunscreens due to their strong ability to filter UVR. The bulk form of TiO_2 and ZnO makes the formulation thick and unaesthetic, which can create white patches on the skin after application. Sunscreen formulators use nanotized forms of these compounds to avoid this nuisance. In addition, inorganic UV filter reflects UV light more effectively than organic filters. In fact, TiO_2 and ZnO nanoparticles allow easy spreading and transparent sunscreen formulations. Nanoparticles are those particles which have a size less than 100 nm. Regardless of having less information about the biological effects of nanoparticles, still they are found in most of the sunscreens. As an example, in Australia, it was evaluated that 30% of UV sunscreens contains ZnO nanoparticles while 70% contain TiO_2 nanoparticles. The small size of nanoparticles is the main concern for their potential toxicity due to which they hide from the immune system and form complexes with protein that can induce ROS generation. The toxicity of particles is related to their surface reactivity. Nanoparticles have more reactive surface area than their bulk counterpart, and thus under UV light, catalyzing reaction are more likely to generate ROS. The main concern about these nanoparticles' toxicity is their use as photocatalysts to generate electricity in photovoltaic cells. Nanoparticles under UV exposure emit electrons which induce free radicals, peroxides, anions, and other ROS generation. Particles' ability to penetrate skin cells depends on their molecular size. Intercellular spaces between the skin cells is 100 nm which can be modified and widened by several topical products (Yang et al. 2007) or after UV exposure-mediated stress.

Various studies have shown the cytotoxic effect of ZnO nanoparticles on several mammalian cell lines, such as on neuroblastoma cells (Brunner et al. 2006), human mesothelioma, rodent fibroblasts (Ghadially et al. 1992), vascular endothelial cells

(Jeng and Swanson 2006), human neural cells, neural stem cells (Lanone et al. 2009), and human alveolar epithelial cells (Gojova et al. 2007). ZnO nanoparticles' toxicity primarily affects the skin barrier after the sunscreen application. ZnO nanoparticles' toxicity can occur at two different levels: cells forming the skin barrier and the tissues where it reached from systemic circulation. In past few years, studies related to ZnO nanoparticles on human dermal fibroblasts have been published. ZnO nanoparticles (20 nm), even at low concentrations, are capable of triggering apoptosis in human dermal, through the p53–p38 pathway**.** The p38 MAP kinases are activated by genotoxic stress and enhance p53 activity by phosphorylation leading to cell cycle arrest or apoptosis. In recent studies, ZnO nanoparticles (60 nm) and bulk (550 nm) phototoxicity were reported in *Caenorhabditis elegans*, a nematode.

Results have revealed that under natural sunlight, bulk and nanotized ZnO phototoxicity are more enhanced, and moreover, ZnO nanoparticles are more important in stimulating phototoxicity than bulk ZnO particles. There is a possibility to decrease undesirable ZnO particles photoreactivity in the sunscreens. These particles are modifiable to a notable context by coating them with organic (such as alkoxy titanates, silanes, and methyl polysiloxanes) or inorganic (such as zirconium, alumina, and silica) elements or doping them by manganese ions (Meyer et al. 2011). Nanoparticles of ZnO are macropinocytosed via skin cell. They stimulate ROS generation in cell cytoplasm. Oxidative stress induces increased p53 and p38 MAPK expressions. The p38 triggers p53 activation through phosphorylation by MAPK. Activated p53 MAPK induced mitochondrial apoptosis pathway in the skin cells. Particle coat made of hydrated oxides can trap hydroxyl radicals and thus can decrease phototoxicity by eliminating or minimizing strong reactivity of photoactivated ZnO particles by quenching and/or reducing ROS generation before their interaction with skin components (Fig. 2).

Biomarkers in Sunscreen-Induced Skin Toxicity

Effectively, biomarkers comprise of tools and technologies that help us to understand the cause of the disease, diagnosis, and treatment of the disease (Mayeux 2004). Biomarkers can be classified into three types: (1) type 0 markers, which correspond to the natural history of the disease, (2) type I markers, which correspond to the mechanism of action of drugs, and (3) type II markers, surrogate markers which correspond to a surrogate endpoint in clinical trials (Malaviya et al. 2006). Skin is the largest organ in the human body, consisting of multiple layers that protect underlying tissues. It consists of several cells such as keratinocytes, melanocytes, fibroblasts, and immune cells which help our body to prevent itself from physical damage, dehydration, pathogen attack, and temperature fluctuations. Skin cells secrete various types of regulatory, structural, and effective molecules, such as cytokines such as IL-1RA, IL-1β, IL-1α, IFN-γ, and TNF-α, chemokines such as CCL-1, CCL-14, CXCL 13, etc. Several studies have found type 0 biomarkers for skin, keratinocytes activity (presence of K16) and inflammatory responses (upregulation of TNF-α and IL-8) (Orro et al. 2014).

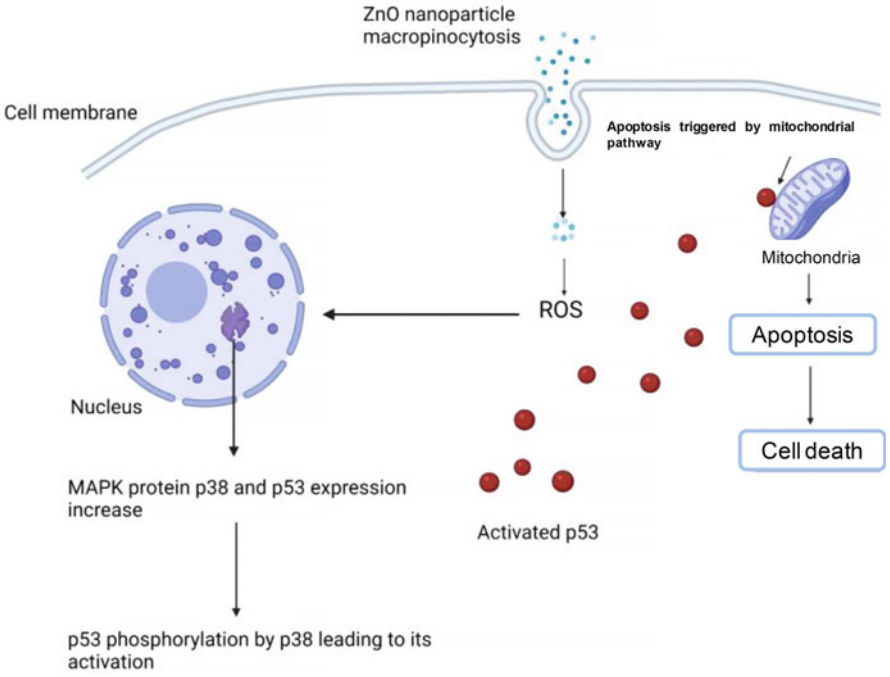

Fig. 2 Schematic representation of a possible toxicity model of ZnO nanoparticles on human epidermal cells. Engulfment of ZnO nanoparticles through plasma membrane of cell involves induction of ROS and activation of p38–p53 MAP kinase pathway. Thereby activated p53 upregulates apoptotic genes through mitochondria and mediating apoptosis

There are several biomarkers known for skin diseases which may also act as a biomarker for sunscreen-induced toxicity. Atopic dermatitis (AD) is correlated with the release of increased concentration of IgE antibodies in the skin (Jung and Stingl 2008). Increased mRNA expression of interferon-γ is also seen in AD patients. Also, an increased level of IL-4 and decreased level of IL-10 was observed in AD patients. Melanoma diagnosis by histological analysis is considered the gold standard but various biomarkers such as KIT gene of mucosal melanoma and BRAF V600E mutation of cutaneous melanoma can also be helpful in the diagnosis of melanoma (Paliwal et al. 2013). Biopsies are the most common method for direct collection of multiple skin layers involved in the removal of the skin strata. Histological analysis of biopsies has been used for the diagnosis of malignancies and abnormalities in biological processes by studying skin fibroblasts. It has several limitations such as its invasive nature and potential tissue degradation. In tape stripping, an adhesive is used which binds to the stratum corneum and removes its contents, which is considered non-invasive and used in the direct sampling of the skin. Usually, an adhesive tape is pressed on to the skin and removed abruptly. It is considered a convenient, rapid, and patient-friendly method. Lack of standardized protocol corresponds to be its primary limitation (Surber et al. 2001). Currently, tape stripping is also used in

isolation of RNA from stratum corneum and analyzed to derive a 312 gene expression signature used for the identification of melanoma (Wachsman et al. 2011).

Sunscreen Filters Tests as Biomarkers

Device-based technologies include microdialysis, microneedle, electroporation, iontophoresis, and ultrasound have been used for sampling of skin's interstitial fluids and the structural components. Iontophoresis and electroporation use electricity to transfer uncharged or charged solutes by electric fields through the skin. Iontophoresis uses a low current usually less than 0.5 mA/cm^2 for a few minutes to 10 min. Electroporation uses high voltage (100 V) for a very short time (μs–ms). Iontophoresis is used for the extraction of various molecules from the skin as well as the extraction of structural components of skin such as prostaglandin E and amino acids. Low-frequency ultrasound can facilitate skin permeabilization with the help of surfactants and extract biomarkers. Ultrasound with the interaction of zwitterionic and nonionic surfactants is shown to harvest several molecules including IgE and cholesterol from the skin. Microdialysis uses a permeable membrane and dialysates for the collection of biomarkers. It is really helpful for continuous monitoring and particularly effective in the case of small hydrophilic molecules such as ascorbic acid, histamines, and growth factors. In situ detection of skin constituents can be done by using Raman spectroscopy (Huang et al. 2011).

Organic UV filters can trigger allergic reactions on the skin. Some of the chemical filters used in sunscreens cause photoallergic contact dermatitis, such as 2-ethylhexyl-p-methoxycinnamate (Parsol MCX), 4-tert-butyl-4′-methoxydibenzoylmethane (avobenzone or Parsol 1789), isoamyl-p-methoxycinnamate (Neo Heliopan® E1000) which can be confirmed by native patch and photopatch testing. Some of the filters also showed delayed hypersensitivities. These tests were used as biomarkers for allergic reactions due to chemical sunscreens (Collaris and Frank 2008). Patch tests are utilized in the identification of the etiologic agent(s) of allergic dermatitis. Photopatch test is used in the detection and identification of certain allergens that cause photodermatitis in a certain individual. First, the skin is exposed to a considerable amount of allergen involved in causing photoallergic contact dermatitis and then the response is recorded with and without light exposure. PABA was earlier used as a sunscreen ingredient, which was found to cause sunscreen photoallergy. In a case study, it was shown that PABA-containing sunscreen applied to the skin caused pruritic, red, papular rash to all photo-exposed sites, and it was confirmed by a positive photopatch test (Waters et al. 2009).

Zinc oxide nanoparticles (ZnO-NPs) are present in various sunscreens. A study conducted in mouse skin showed that ZnO-NPs are capable of generating reactive oxygen species (ROS) and in combination with UVB generated an even higher amount of ROS. Excessive generation of ROS resulted in cutaneous pathological conditions. ZnO-NPs elicit a photocatalytic response in the dermal layer and causes skin cells to form ROS. Excessive ROS can have various effects on the cells by oxidative stress and can lead to DNA damage including DNA strand breaks,

deletion, mutations, and formation of 8-OHdG. DNA damage can be used as a biomarker through detection by a sensitive technique, alkaline comet assay, which detects single- and double-stranded breaks and alkali label sites. γ-H2AX is part of the subfamily H2AX of histone proteins which are found in early events where double-stranded DNA breaks are introduced. γ-H2AX is considered as a sensitive marker of double-stranded breaks and was also increased when cells were exposed to ZnO-NPs and UVB (Pal et al. 2016).

Oxidative Stress Biomarkers and Their Role in Sunscreen-Induced Skin Toxicity

Oxidative stress is a situation of disequilibrium between oxidant generation and antioxidant defense, where oxidant activity accelerates the neutralizing capacity of antioxidants. This is related to diverse pathological conditions such as age-related diseases, cardiovascular diseases, atherosclerosis, diabetes mellitus, and cancer. Oxidative stress is recognized as a primary action in the toxicity of various chemicals as well as in the diagnosis of various diseases (Ghezzi 2020). ROS generation involves oxygen and nitrogen free radicals formation that lead to oxidative stress in the body. Increased amount of free radicals leads to cell injury. ROS includes hydrogen peroxide, hydroxyl radicals, singlet oxygen, nitric oxide (NO), superoxide anion radical, and peroxynitrite. Sunscreens have been widely used for prevention against ultraviolet solar radiation for more than a century. In normal physiological condition, ROS production is generally at a reduced level and is cleared by internal antioxidant system which includes catalase, superoxide dismutase (SOD), glutathione peroxidase, and some low molecule substances such as vitamin E and vitamin C. Vitamins, such as vitamin B3 and vitamin D3, also safeguard from other closely associated oxidative stress pathway. A biomarker in a biological system can be measured in the diagnosis, to predict the onset or development of a disease process.

The definition of biomarkers is intentionally broad and the application of biomarkers can be used for the identification of specific genes, proteins, or other products of cellular and biological processes as well as the response of cells or tissues for therapeutic application (Maiese et al. 2010). Interestingly, additional benefit of some biomarkers is to function as carrier markers for the prediction of clinical results in some cases. For example, levels of estrogen as a biomarker may predict the onset of postmenopausal diseases such as breast cancer and a poor clinical outcome.

The major cellular target of oxidative stress relays on the cell type, behaviour of the stress on the site of formation and proximity of ROS to a specific target, and the amount of stress. Usually, ROS have short half-lives (Karthick et al. 2016). Therefore, to detect ROS, in vivo special techniques are necessary. Oxidative stress biomarkers can determine the extent of oxidative injury and also indicate the source of the oxidant. For predicting the consequences of oxidation, oxidative stress biomarkers are very essential and are divided into two major categories: (a) generation of modified molecules by ROS and (b) utilization or induction of enzymes or antioxidants (Fig. 3).

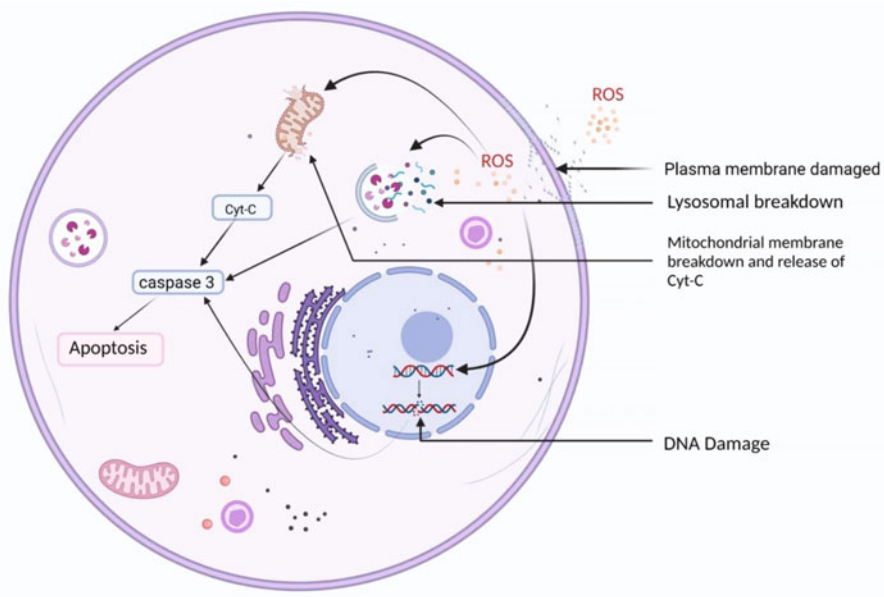

Fig. 3 ROS induced organelles mediated programmed cell death or apoptosis. Oxidative stress mediates ROS induction through external photosensitizers. This ROS is then responsible for lysosomal and mitochondrial destabilization, DNA damage, and leads to programmed cell death or apoptosis

Assessment of these biomarkers in the body fluids such as blood, urine, cerebrospinal fluid, bronchoalveolar lavage fluid) or breath condensate empowers monitoring of the oxidative stress status repeatedly in vivo, which is difficult with invasive analysis. It should be appropriate to evaluate the levels of oxidative stress markers along with routine laboratory assessments. In human liver cells, detection of ROS generation might be possible by the use of fluorescent probes (Yamada et al. 2020). Sunscreens are organic or inorganic filter substances for topical application on the skin (Pinto et al. 2022). They were originally developed for prevention of solar erythema. Since other detrimental effects of ultraviolet radiation (UVR) have been identified such as skin aging, cutaneous immunosuppression, and carcinogenesis, sunscreen users and dermatologists expect sunscreens to be efficient also against these problems. With increasing evidence of anti-inflammatory and antitumor effects of vitamin D that is synthesized in the skin, a potential risk of inducing vitamin D deficiency by sunscreen use has also been discussed.

Oxidative Stress Biomarkers and Apoptosis

Particularly keratinocytes, in the skin, are constantly exposed to various types of stress, including sunlight and oxygen in the air. Ultraviolet (UV) irradiation is one of the major sources of ROS. UVB photons, which have an energy greater than UVA

photons, tend to damage the skin even after a short exposure (Borowiak et al. 2015). Previous studies have demonstrated that UVB photons induce various types of DNA damage, such as pyrimidine dimers and (6–4) photoproducts, and generate 8-hydroxy-2′-deoxyguanosine (8-OHdG) through ROS in mouse, as well as in human keratinocytes (Hayden et al. 2005). Fifty percent of the UV-induced damage is presumably due to the formation of free radicals. The role of singlet oxygen ($^1O_2$) has been recorded among the ROS, that entails genetic changes and cell damage through photodynamic reactions. ROS and damage of DNA have been clearly linked to apoptotic cell death processes. Apoptosis is a process of programmed cell death that can be initiated by oxidative stress and DNA damage with the involvement of mitochondria and lysosome. Apoptosis is mainly triggered by two pathways: extrinsic and intrinsic. In the extrinsic pathway, cell death occurs through interaction of receptors with ligands which are mitochondrial dependent. However, the intrinsic pathway is activated by internal stimuli such as oxidative stress, DNA damage, as well as UVR through mitochondria (Dubey et al. 2019).

The mitochondrial-dependent apoptotic pathway is primarily controlled and regulated by Bcl-2 proteins, which are members of both anti- and proapoptotic families. During apoptosis, apoptotic stimuli, Bax is activated and gets translocated to mitochondria from cytosol and facilitates mitochondrial membrane permeability (Fig. 4).

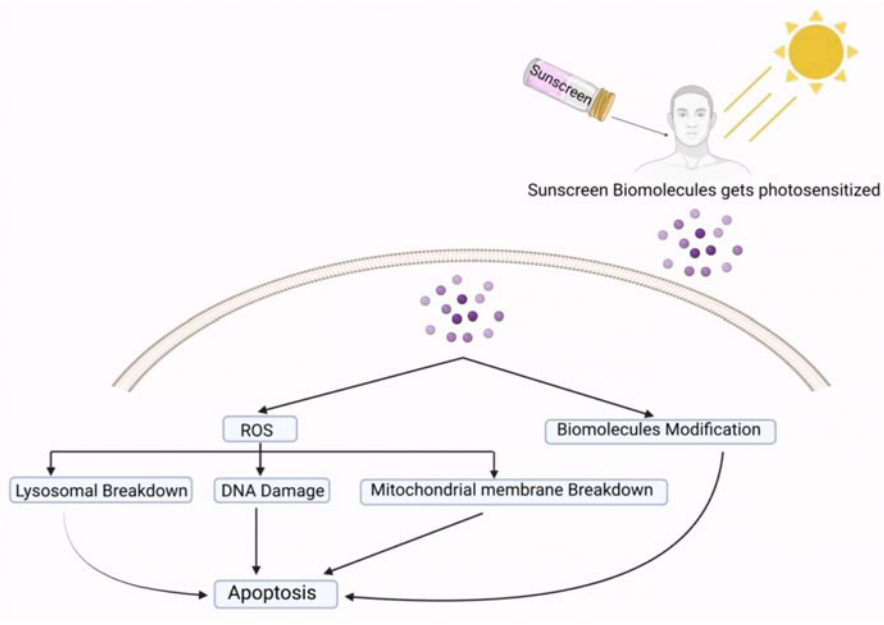

Fig. 4 UVR-mediated photosensitized sunscreen/sunscreen ingredients depicting ROS generation and apoptosis. Components of sunscreens get photosensitized in the presence of UVR/sunlight and generate ROS that is involved in multiple organelles (mitochondrial and lysosomal) membrane damage and DNA damage that leads to apoptosis. These may also cause biomolecule modification like lipid peroxidation

Studies linked the role of mitochondria and lysosomes in UVB-mediated apoptotic cell death. It has been established that lysosome membrane destabilization and release of its content into the cytosol may initiate mitochondrial-dependent lysosomal apoptosis pathway. Thus, excessive UVB exposures accelerate the DNA damage via photooxidation. Sunscreens' active ingredient causes tumor initiation and transcriptional modification of tumor-promoting genes. Sunlight in dermal phototoxicity is a growing aim for human health due to ozone layer reduction, which results in increased radiation, particularly, UVB at the surface of the earth. UVB exposure is more dangerous, causing cell organelle damage; the contribution of UVB is around 80% towards sunburn, while the contribution of UVA is 20%. UVB is linked to tissue injuries, inflammatory skin damages, DNA damage, oxidative stress, skin aging, cancer, and cell death. p-Phenylenediamine is a photosensitized ingredient that leads to apoptosis through the dysfunction of mitochondria and lysosome. JNK/p38 activation was considered to be involved in various forms of cell death in response to a diversity of cellular injuries. MAP kinase (JNK and p38) are involved in apoptotic cell death which elevates levels of ROS and initiates UVB-induced signaling pathways (Chen et al. 2018). p38 and JNK activate apoptotic cell death signaling by upregulation of mitochondrial proapoptotic protein and antiapoptotic proteins by various phosphorylation events. JNK immediately translocated to mitochondria. Cytochrome C release from mitochondria activates caspase cascade that initiates apoptosis. Antiapoptotic proteins are inactivated by JNK while activating proapoptotic Bcl-2 family members.

Pro-inflammatory Markers and Their Role in Sunscreen-Induced Toxicity

The skin, the largest organ provides a protective barrier against microorganisms or any harmful organisms and substances, protects our body against UVR from the sun, and regulates the body temperature. So, various cosmetics and personal care products are used to enhance beauty, cleanse, making more attractive, preventing skin tanning, etc. However, the skin is not just a barrier. It has other roles such as protection from external danger, maintains hydration, and also metabolizes toxins. The cosmetics include a lot of beauty ranges: sunscreens, lipsticks, hair dyes, perfumes, makeup materials, etc., in which the chemicals present cause an adverse effect on the skin (Mujtaba et al. 2021). Most sunscreens and their constituents available in the markets absorbs radiation in the range of UVA (320–400 nm), UVB (290–320 nm), or in both spectra, e.g., octyl salicylate, octyl methoxycinnamate, etc., absorbs in UVB range; benzophenone-3 absorbs in both UVA and UVB ranges. However, adverse effects of sunscreens have shown vigorous toxicity, phototoxicity, photogenotoxicity in ocular and cutaneous screening. In spite of regulatory authority guidelines, there are a lot of published reports of bad effects of sunscreens on human skin (Keyes et al. 2019). Research demonstrated that active sunscreen formulations and their ingredients like 2,4-dichlorophenoxyacetic acid enhance dermal penetration across the nude mouse skin as well as human skin.

The aging and inflammation of skin cells increase after exposure, and many sunscreens are used nowadays to slow it down. Many biological mechanisms such as the release of cytokines, degradation of proteins, or decrease in extracellular matrix protein synthesis altered in skin aging can be used as a marker of aging (Lago and Puzzi 2019). The alarmins, other endogenous molecules, are critically important in injury response to chemicals or sunscreens, as well as pro-inflammatory cytokines (e.g., TNF-α and IL-1β) and high mobility group (HMG) transcription regulatory proteins have been used to characterize as well as identify the toxicity-mediated inflammations of the skins (Fig. 5). In healthy skins, the exposure to numerous toxins and application of sunscreens may cause the initiation of inflammatory responses which leads to accumulation of monocytes and macrophages, followed by their accumulation that phagocytose dead cells, debris, and also induces injured cells to undergo apoptosis.

Increasing research shows that toxin-induced dermal injury causes inflammations as well as causes aberrant changes at the genetic level and in epigenetic factors. However, defined and distinct biomarker pattern of dermal toxicity is a challenge. A significant emerging factor as a biomarker of toxicity and inflammations for the recognition of dermal toxicity can be injury-related alterations in the tissues.

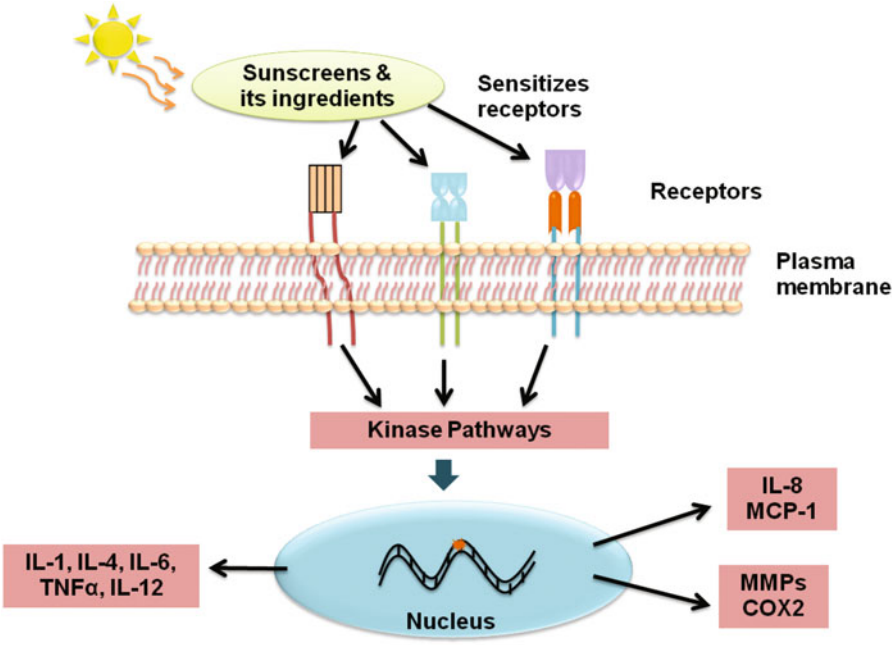

Fig. 5 Sunscreen ingredients sensitization and activation of downstream inflammatory biomarkers. Sunscreens and their ingredients are photosensitized in the presence of sunlight/UVR and activate inflammatory pathways that involve cytokines and chemokines. These inflammatory response molecules can be used as biomarkers and measuring their levels can give a view of damage caused by sunlight/UVR exposure

However, new studies are on the way to reveal the complex network of interactions via identifying biomolecules released in the inflamed and injured skin tissues. The increasing knowledge of identified specific subsets of biomarkers for inflammation and dermal toxicity will reflect biochemical activities underlying various cellular damage effects, injury healing, response to water loss and pH variations, inflammatory responses and inflammations along with tissue repair and regeneration.

Inflammation Identifiers as Biomarkers

The identification of dermal toxicity as well as inflammation biomarkers has been advanced by the development of high-throughput technologies, which focus on finding toxicity and inflammation responsive genes at the whole genome level and other biochemicals, transcriptomes, metabolomes, cytokines, chemokines, etc. These biomarkers are used for the recognition of dermal injury, inflammations, and as an indicator of sensitivity and toxicity by sunscreens (Fig. 6). Thus, the main purpose of dermal biomarkers is to test the potential toxicity and inflammatory risk with substance exposures. Recent work evidences that analysis of biomarkers will increase the understanding of the molecular mechanism that links skin diseases, injury, and inflammations. Therefore, the identification of proteins, mediators,

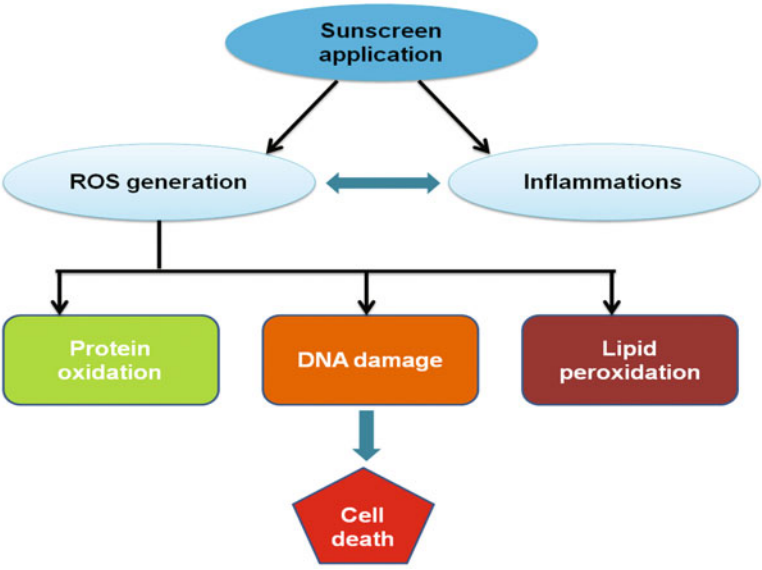

Fig. 6 Sunscreen application involves different cell-mediated toxicity pathways. The multitude of effect of sunscreen application entails oxidative stress, ROS generation, and inflammatory responses. These responses are capable of biomolecule modification (such as protein oxidation, lipid peroxidation, etc.) to induce membrane potential loss in cell membrane, organelles membrane, and DNA damage that eventually marks the apoptosis or cell death

transcription factors, etc., as a biomarker for toxicants has become the most intense area of research (Lionetto et al. 2012).

Many markers can be identified as biomarkers on the basis of ideal characteristics as: appropriate half-life, sensitivity, precision, specificity for the toxin, low cost, associated with health and quantitative relationship with exposures to toxins, sunscreens and its constituents. Conversely, these biomarkers are sunscreens toxicity specific and may also be used in continuous regenerating skin tissue injury. Therefore, the classification of dermal biomarkers of toxicity for injury and inflammations is important, not only for the identification of toxicant substances but also for appropriate injury or inflammations caused.

The chemical-induced burns result in biochemical communications within the injured skin tissue to release inflammatory biomediators, e.g., nitric oxide, prostaglandins, histamine, thromboxane, etc. These increase blood capillary permeability and lead to localized wound inflammation and edema. The cell membrane dysfunction and damage leads to changes in sodium ATPase activity. Change in sodium ATPase activity causes shift between sodium and membrane-derived mediator and initiates vasoactive and inflammatory mediators release. These mediators are further responsible for local vasoconstriction, systemic vasodilatation, and increase transcapillary permeability. A rapid transfer of water, inorganic solutes, and plasma proteins between intravascular and interstitial spaces occur due to increased permeability of transcapillaries. Biomarkers of barrier loss of damaged stratum corneum are the most significant molecules that involve changes in protein and gene expression of filaggrin, profilaggrin, keratinocytes flattening regulator for barrier formation and regulators of calcium homeostasis, released amino acids upon degradation of proteins and water-retaining barrier components such as cytokeratin 1, 10, and 11, desmoglein-1, pyrrollidone carboxylic acid, adhering junction proteins desmocollin-1 and corneodesmosin, and long-chain ceramides.

The release of histamine, from mast cells in response to allergens and toxic irritants, evokes itch and skin sensations. The best-defined biochemical marker for histamine release in the presence of toxins is the serum basophil histamine release (SBHR) assay (Gewehr et al. 2013). Markers of apoptosis or inflammation or debridement by chemicals or other toxic or sunscreen skin injury cause the breakdown of cellular membranes resulting in release of intracellular proteins into the extracellular environment and these molecules show chemoattraction and immune modulation potency as well as recruiting, activating immune cells at the site of injury. They also release additional proteins, pro-inflammatory cytokines, reactive mediators, preventing microbial invasion and also prepares injured dermal tissue to regenerate surface for repopulation.

Application to Prognosis

Dermatologists warn patients about skin damage caused due to UV rays and they suggest using sunscreen products with high SPF to evade side effects of UVR and the development of skin cancer. However, increased usage of sunscreen products in

consumers' lives questions sunscreen safety in several studies. Several studies present evidence about the harmful effects of sunscreens in skin cells. Different methods and models are being used to test the toxicity of the concerning UV filters. Better and improved standardized methods are needed for sunscreen toxicity studies. The main focus of sunscreen formulators should be on UV filter carriers as they can prevent penetration of UV filters in the skin (Meyer et al. 2011), whereas some of the carriers promote the opposite. Alarming the consumers about sunscreen toxicity is not necessary, because it may cause a prohibition of products that protects the consumers from skin cancer and may lead to a surge in skin cancer cases. It may create a sunscreen phobia among the consumer and increase the exposure to UVR which may ultimately lead to more cases of melanomas and other skin diseases.

Mini-Dictionary

1. Atopic dermatitis – A chronic skin condition characterized by patches of dry, inflamed, and itchy skin.
2. Biomarker – A biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease.
3. Biomediators – A biological sensing element (an enzyme, a tissue, living cells, etc.) that interacts with specific analytes producing various kinds of signals.
4. Contact dermatitis –A red, itchy rash caused by direct contact with a substance or an allergic reaction to it.
5. Inflammation – A process by which your body's white blood cells and the things they make protect you from infection from outside invaders, such as bacteria and viruses.
6. Melanoma – A form of skin cancer that begins in the cells (melanocytes) that control the pigment in your skin.
7. Mesothelioma – Mesothelioma is a type of cancer that develops from the thin layer of tissue that covers many of the internal organs.
8. Micropinocytosis – The engulfment and uptake process of large amounts of fluids and membranes.
9. Photoallergy – An allergic reaction caused by drugs in which ultraviolet exposure changes the structure of the drug so that it is seen by the body's immune system as an invader.
10. Photogenotoxicity – A genotoxic response observed after exposure to a chemical photoactivated by UV or visible light.
11. Photoproducts – Any product which is produced by a chemical reaction that uses light or other electromagnetic radiation to increase energy of particles.
12. Photoprotection – The reduction of ultraviolet (UV) radiation-induced skin damage and skin cancers.
13. Photosensitization – The process of initiating a reaction through the use of a substance capable of absorbing light and transferring the energy to the desired reactants.

14. Phototoxicity – Chemically induced skin irritation, requiring light, that does not involve the immune system.
15. Sensitizer – A substance that causes exposed individuals to develop an allergic reaction in normal tissue after repeated exposure to the substance.

Key Facts of Sunscreens: Toxic Effect as Determined by Biomarkers

- UVA is responsible for wrinkles, premature aging, deep damage to the skin, and potential skin cancer, and UVB rays hurt your eyes and burn your skin.
- Skin accounts for 15% of your body weight and renews its outer layer, the epidermis every 28 days.
- Australia is known for having the highest rate of skin cancer in the world and most of it is preventable by applying sunscreen on the sunlight-exposed skin.
- Reactive oxygen species (ROS) is not always harmful; in fact, it is beneficial to the cell, supports cellular processes and viability.
- UV rays can react with melanin and melanin also assists the absorption of UV rays, causing serious damage to skin as in the case of sunburn.
- Both humans and wildlife are affected by UV rays and a long duration of UVB exposure slows the growth of almost all green plants.

Summary Points

- Most sunscreens and their constituents available on the markets absorb radiation in the range of UVA (320–400 nm), UVB (290–320 nm), or in both spectra, e.g., octyl salicylate, octyl methoxycinnamate absorbs in the UVB range; benzophenone-3 absorbs in both UVA and UVB ranges.
- However, adverse effects of sunscreens have shown vigorous toxicity, phototoxicity, photogenotoxicity in ocular and cutaneous screening.
- In spite of regulatory authority guidelines, there are a lot of published reports of the bad effects of sunscreens on human skin.
- Research demonstrated that active sunscreen formulations and their ingredients like 2,4-dichlorophenoxyacetic acid enhance dermal penetration across the nude mouse skin as well as human skin.
- Organic UV filters are chemical compounds that can cross the skin barrier because of their low molecular weight and lipophilic nature, and are known to be allergenic.
- Organic filters are considered to have higher chances to induce toxicity than inorganic filters.
- Majority of studies agreed to photo-instability of organic UV filters leading to deleterious effects by ROS formation in skin cells.

- The concern regarding the inorganic UV filters such as TiO_2 and ZnO is their capability to cross the skin barrier.
- Markers of barrier loss, stratum corneum damage, and other biochemical markers for the same act as an indicator of the cost of damaging the physical barrier that protects it from external dangers.
- Biological mechanisms like cytokines release, protein degradation, or extracellular matrix protein downregulation can be exploited as skin aging biomarkers.

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Drug-Induced Nephrotoxicity and Use of Biomarkers

36

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Abstract

Nephrotoxicity has been defined as renal injury that is caused due to direct or indirect exposure, mostly to drugs and other exogenous industrial or environmental chemicals. Exogenous toxicants like drugs or endogenous toxicants like metabolic products of drugs cause damage in the kidney, and due to these, the detoxification processes and excretion do not work in a proper way and this ultimately leads to nephrotoxicity. It becomes very important to understand the mechanisms of these toxicities as it can provide us with very useful information that can further help us in the development of safe drugs. Drug-induced nephrotoxicity can be attributed to changes in hemodynamics of glomerulus, toxicity of tubular cells, inflammation, rhabdomyolysis, nephropathy, and thrombotic microangiopathy. Early detection of nephrotoxicity is the key for its proper management. In this regard, work has been done by various scientists on different biomarkers: traditional ones like glomerular filtration rate, albumin, creatinine, blood urea nitrogen and novel ones like neutrophil gelatinase-associated lipocalin, kidney injury molecule 1, liver-type fatty acid-binding protein, calprotectin, brain natriuretic peptide, soluble ST2, galectin-3, urinary microRNA, β2-microglobulin, cystatin C, and clusterin which can be used to access the nephrotoxicity. In this chapter, we have listed the drugs that are responsible for nephrotoxicity and summarized mechanisms of drug-induced nephrotoxicity, potential biomarkers, diagnosis, diseases, and treatment for the nephrotoxicity.

Keywords

Biomarkers · Drug toxicity · Cell toxicity · Inflammation · Nephrotoxicity · Renal health · Creatinine · Nephritis · Glomerular filtration rate · Blood urea nitrogen · Albumin · miRNA

Abbreviations

ACE	Angiotensin-converting enzyme
ACEI	Angiotensin-converting enzyme inhibitors
ACR	Albumin-creatinine ratio
ADH	Antidiuretic hormone
AIN	Acute interstitial nephritis
AKI	Acute kidney injury
ARB	Angiotensin receptor blocker
ARB	Angiotensin receptor blockers
ARF	Acute renal failure
ATN	Acute tubular necrosis

BUN	Blood urea nitrogen
CG/MS	Gas chromatography mass spectrometry
CIN	Chronic interstitial nephritis
CKD	Chronic kidney disease
CN	Crystal nephropathy
DCT	Digital convoluted tubule
DN	Diabetic nephropathy
DRT	Distal renal tubular
ELISA	Enzyme-linked immunoassay
EMEA	European Medicines Evaluation Agency
ESRD	End-stage renal test
FDA	Food and Drug Administration
GAA	Guanidinoacetic acid
GAMT	Guanidinoacetate methyltransferase
GFR	Glomerular filtration rate
GN	Glomerulonephritis
IGFBP	Insulin-like growth factor-binding protein
KDIGO	Kidney Disease Improving Global Outcomes
KIM	Kidney injury molecule
L-FABP	Liver-type fatty acid-binding protein
MMP	Matrix metalloproteinase
NGAL	Neutrophil gelatinase-associated lipocalin
NSAID	Nonsteroidal anti-inflammatory drugs
OAT	Organic anion transporters
OCT	Organic cation transporters
PCT	Proximal convoluted tubule
PRT	Proximal renal tubular
RAS	Renin-angiotensin system
RT-PCR	Reverse transcriptase polymerase chain reaction
TEC	Tubular epithelial cells
TIMP	Tissue inhibitor of metalloproteinases
UAG	Urinary angiotensinogen

Introduction

Important functions of the body like acid base homeostasis maintenance, reabsorption of nutrients, regulation of the extracellular environment, regulation of osmolality, blood pressure regulation, detoxification, and excretion of toxic metabolites and drugs in the form of urine are done by one of the main organs of the body, i.e., kidney. The kidney is particularly liable to drug injury due to the mentioned reasons: (1) It receives 20–25% of the resting cardiac output, so kidney gets exposed to the circulating drug more than other organs. (2) As kidney tubules are responsible to concentrate the filtrate, it makes them more exposed to drugs. (3) As the tubules have high energy requirements due to filtration process, they become more prone to nephrotoxic injury

(Griffin et al. 2019). Major kidney issues that can arise due to drug toxicity are nephritis, nephrosis, intrarenal obstruction, acid-base, and fluid electrolyte imbalance disorders that may lead to acute renal toxicity. Out of the total nephrotoxicity cases, around 20% are caused by the use of drugs (refer to Table 1). In addition, as the elderly are generally on one or other medication, this further enhances the incidence of nephrotoxicity up to 66%. Drug-induced nephrotoxicity inclines to occur more recurrently in patients with comorbidities like diabetes, chronic kidney disease, heart diseases, etc. Hence, early detection of adverse reactions of drugs becomes important to avert development to acute renal disease (Assadi 2015).

There are many probable mechanisms of renal injury caused due to drugs; for example, anti-TB drug rifampicin is known to cause renal injury by mechanism of acute/chronic interstitial nephritis (Beebe et al. 2015). According to a study done by Chang et al. (2014) in aging patients on anti-TB treatment from 2006 to 2010, 99 out of 1394 patients i.e., 7.1%, had reported acute renal injury. For diagnosis of nephrotoxicity traditionally, a simple blood test is done generally whereby parameters like blood urea nitrogen (BUN), serum creatinine concentration, glomerular filtration rate, and urine analysis are measured. There are many factors which further enhance the nephrotoxicity like combination of drugs, age of the patient,dose and duration of the drug and any co-morbidity (Refer to Fig. 1) Discovery and

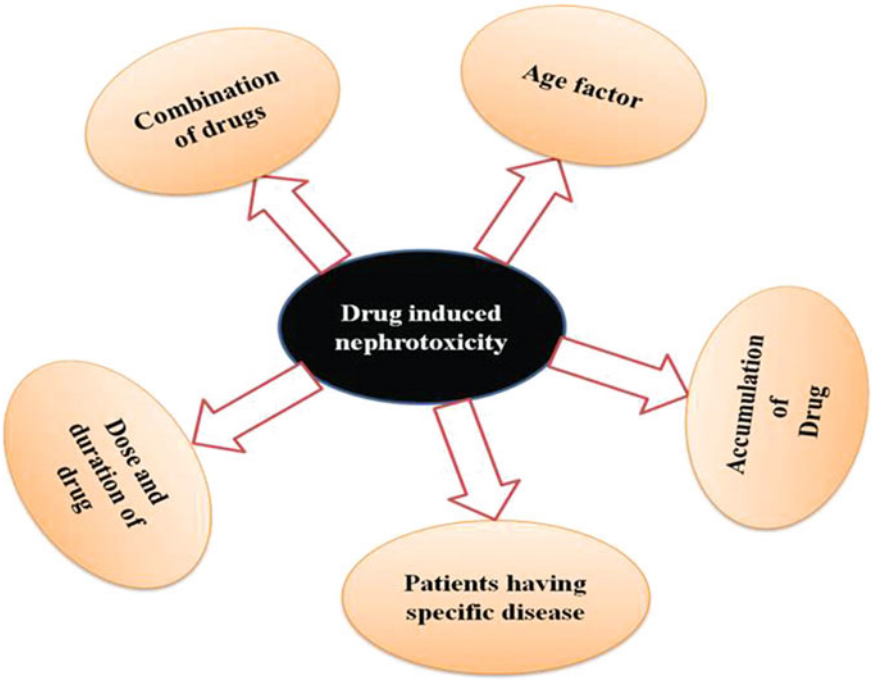


Fig. 1 Factors associated with increased risk for nephrotoxicity. This figure illustrates different factors responsible for drug-induced nephrotoxicity.

development of biomarkers that can detect kidney dysfunction at the early stage are need of the hour.

Mechanisms of Drug-Induced Nephrotoxicity

A variety of factors, like molds and fungi; cancer medications such as cisplatin; antibiotics; drugs as mentioned in Table 1; metals like mercury, arsenic, and lead; etc., leads to nephrotoxicity which involves changes in the structure of nephron. Drugs which cause nephrotoxicity works through a number of mechanisms which induces an alteration in the physiological and biochemical environment of various regions like change in the hemodynamics of glomerulus, inflammation, tubular cell toxicity, crystal nephropathy, rhabdomyolysis, and thrombotic microangiopathy (Ferguson et al. 2008). Millions of nephrons are present in kidney which filter waste from the blood, sustain liquid equilibrium of the body, and regulate blood pressure. Any kind of nephrotoxicity leads to loss of an adequate amount of cells of any part of the nephron, and particularly, proximal tubules are the principal target of majority of nephrotoxicants. Following headings explain the different mechanisms of nephrotoxicity.

Changes in Glomerular Hemodynamics

It is well known that in a healthy and grown-up individual, glomerular filtration rate (GFR) is 120 mL/min. GFR is a measure of proper functioning of kidneys which works to clean the blood by taking out any excess of water and waste. Kidneys work through an auto-regulatory mechanism which regulates and maintain the intraglomerular pressure by filtrating the fluid entering through afferent arterioles. The renin-angiotensin system (RAS) is a well-studied system which maintains the blood pressure and fluid balance by conversion of inactive prorenin to Angiotensin I which is further converted to Angiotensin II under the effect of angiotensin-converting enzyme. Angiotensin II acts as vasoconstrictor resulting in increased blood pressure. On the other hand, prostaglandins are used to vasodilate and expand the afferent arterioles. Any medication which falls in the category of non-steroidal anti-inflammatory drugs [NSAIDs] act as anti-prostaglandin drugs or show anti-angiotensin activity such as angiotensin-converting enzyme inhibitors or angiotensin receptor blockers effects activity of prostaglandin and angiotensin and hence blocks the elevation of blood pressure leading to toxicity in glomerulus (Schoolwerth et al. 2001; Palmer 2002).

Tubular Cell Toxicity

Continuous exposure of drugs in the tubular part of nephron during the process of reabsorption in the glomerulus leads to the tubular toxicity, and as proximal convoluted tubules are maximum involved in reabsorption, they are affected adversely (Perazella 2005). The major factors which lead to cytotoxicity are generation of free radicals that

Table 1 List of different drugs showing nephrotoxicity. This table lists all the drugs that are responsible to cause nephrotoxicity. This table has been self-prepared by the authors from different literature sources and thus there are no copyright issues

Drug category	Medication	Nephrotoxicity
Anti-arthritic	Gold sodium thiomalate	Nephrotic syndrome, glomerulonephritis
Anti-fungal	Amphotericin B	ATN, DRT acidosis
Anti-histamine	Diphenhydramine	Rhabdomyolysis
Anti-hypertensive	Angiotensin-converting enzyme inhibitors (ACEI)	Acute kidney injury
	Angiotensin receptor blockers (ARB)	Acute kidney injury
Anti-microbial	Aminoglycosides	ATN
	Beta lactams	AIN
	Carbenicillin	Metabolic alkalosis
	Cephalosporin	ATN
	Pentamidine	ATN
	Puromycin	Nephrotic syndrome
	Quinolones	AIN, CN
	Rifampin	AIN
	Sulfonamides	AIN, CN
	Tetracycline	ATN
	Vancomycin	AIN
Anti-neoplastic	Cisplatin	CIN
	Cyclophosphamide	Hemorrhage cystitis
	Interferon-α	Glomerulonephritis
	Methotrexate	CN
	Mitomycin-C	Thrombotic microangiopathy
Anti-platelet	Clopidogrel	Thrombotic microangiopathy
Ant-ipsychotic	Haloperidol	Rhabdomyolysis
	Lithium	CIN, glomerulonephritis, rhabdomyolysis
Anti-rheumatic	D-penicillamine	Nephrotic syndrome
Anti-viral	Acyclovir	AIN
	Ganciclovir	CN
	Indinavir	AIN, CN
Bisphosphonate, osteoporosis prevention	Pamidronate acid	Glomerulonephritis
Carbonic anhydrase inhibitor	Acetazolamide	PRT, acidosis
Contrast medium	Contrast agents	ATN
Corticosteroid	Cortisone	Hypertension, metabolic alkalosis
Gastrointestinal	Cimetidine	AIN

(continued)

Table 1 (continued)

Drug category	Medication	Nephrotoxicity
	Ranitidine	AIN
Hypoglycemic	Phenformin	Lactic acidosis
Hypouricemic agent	Allupurinol	AIN
Immunosuppressive	Cyclosporine	ATN, CIN, thrombotic microangiopathy
	Tacrolimus	ATN
Lipid-lowering	Statins	Rhabdomyolysis
Diuretic	Furosemide	AIN
	Azides	AIN
Muscle relaxant	Quinine	Thrombotic microangiopathic
Narcotic analgesic	Cocaine	Rhabdomyolysis
	Methadone	Rhabdomyolysis
Non-narcotic analgesic	Acetaminophen	ATN, CIN
	Aspirin	CIN
	Phenacetin	CIN
Nonsteroidal anti-inflammatory	Naproxen	AIN, CIN, ATN, glomerulonephritis
Penicillin	Penicillin G	Glomerulonephritis
Proton-pump inhibitor	Lansoprazole	AIN
	Omeprazole	AIN
	Pantoprazole	AIN
Psychostimulant	Methamphetamine	Rhabdomyolysis
Sedative – hypotonic	Benzodiazepines	Rhabdomyolysis
Sulfonylureas	Chlorpropamide	Inappropriate ADH secretion, hyponatremia
Uricosuric	Probenecid	Nephrotic syndrome, CN

Abbreviations: *AIN*-acute interstitial nephritis, *ATN*-acute tubular necrosis, *PRT*-proximal renal tubular, *CIN*-chronic interstitial nephritis, *DRT*-distal renal tubular, *CN*-crystal nephropathy

shoot the oxidative stress, damage mitochondrial structure in tubules, and alter the tubular transport (Markowitz et al. 2003; Markowitz and Perazella 2005). The various drugs like aminoglycoside antibiotics, anti-fungal medicines such as amphotericin B, anti-cancer medications like cisplatin, and foscarnet incite cytotoxicity.

Inflammation

Another mechanism via which nephrotoxic drug work is inflammation. Inflammation in different parts of nephron such as glomerulus and proximal convoluted tubules and their adjoining regions such as cell matrix has been observed, followed by fibrilization of kidney tissue. The aftermath effects of inflammation are induction of toxicity, glomerulonephritis, and intestinal nephritis. Glomerulonephritis which

encompasses a range of disorders leads to inflammation of glomeruli, and the situation is worsen by the presence of other factors like autoimmune disorders, cancer, etc. It is closely related to proteinuria (Perazella 2005) as there are inflammatory symptoms leading to higher cellular proliferation and an alteration in the basement membrane of glomerulus. It has been found that nephrotoxicants can stimulate and lead to different glomerular diseases like diabetes, obesity, hypertension, and systemic lupus erythematosus (Turner et al. 2006; Nozaki et al. 2012). Also, it leads to renal pathologies such as acute interstitial nephritis which is induced by NSAIDs frequently used as pain relievers and antibiotics (Rossert 2001) and chronic interstitial nephritis which arises due to constant long-term use of anti-cancerous drugs, proton-pump inhibitors, etc. (Isnard et al. 2004; Rodriguez-Iturbe and Garcia-Garcia 2010).

Crystal Nephropathy

Crystal nephropathy refers to renal parenchymal deposition of crystals leading to kidney injury. Crystal nephrotoxicity is induced due to the precipitation of different drugs (Yarlagadda and Perazella 2008; Mulay and Anders 2017). Presence of poorly soluble drugs like atazanavir used for the treatment of patients with immunodeficiency, protease inhibitors and sulphadiazine used for the treatment of cerebral toxoplasmosis, antibiotics (ampicillin, ciprofloxacin), and antiviral agents (acyclovir foscarnet, ganciclovir, indinavir; methotrexate; and triamterene) leads to crystallization in urine (Markowitz and Perazella 2005; Perazella 2005). Apart from these drugs, about 20 other molecules, such as ceftriaxone or ephedrine-containing preparations in patients taking high doses or long-term usage or patients taking unrestrained calcium/vitamin D supplements, or being exposed to carbonic anhydrase inhibitors like acetazolamide or topiramate, aggravate the situation (Daudon et al. 2018).

Rhabdomyolysis

Rhabdomyolysis is a syndrome in which muscle fiber contents are discharged into the bloodstream when skeletal muscle injury leads to lysis of myocyte thereby releasing contents like myoglobin and creatinine kinase in the mainstream. The release of intracellular content like myoglobin incites renal injury leading to tubular toxicity and obstructions which effect the filtration process of nephron (refer to Fig. 2), hence a change in GFR is found (Coco and Klasner 2004). There are multiple drugs known for causing rhabdomyolysis with statins recognized ones, and others in the list are heroin, methadone, methamphetamine, ketamine (Ketalar), and another reason is alcoholism (Huerta-Alardín et al. 2005; Naughton 2008).

Thrombotic Microangiopathy

Thrombotic microangiopathy is due to an organ damage which can be caused by inflammation, platelet thrombi in the microcirculation, or due to toxicity in the

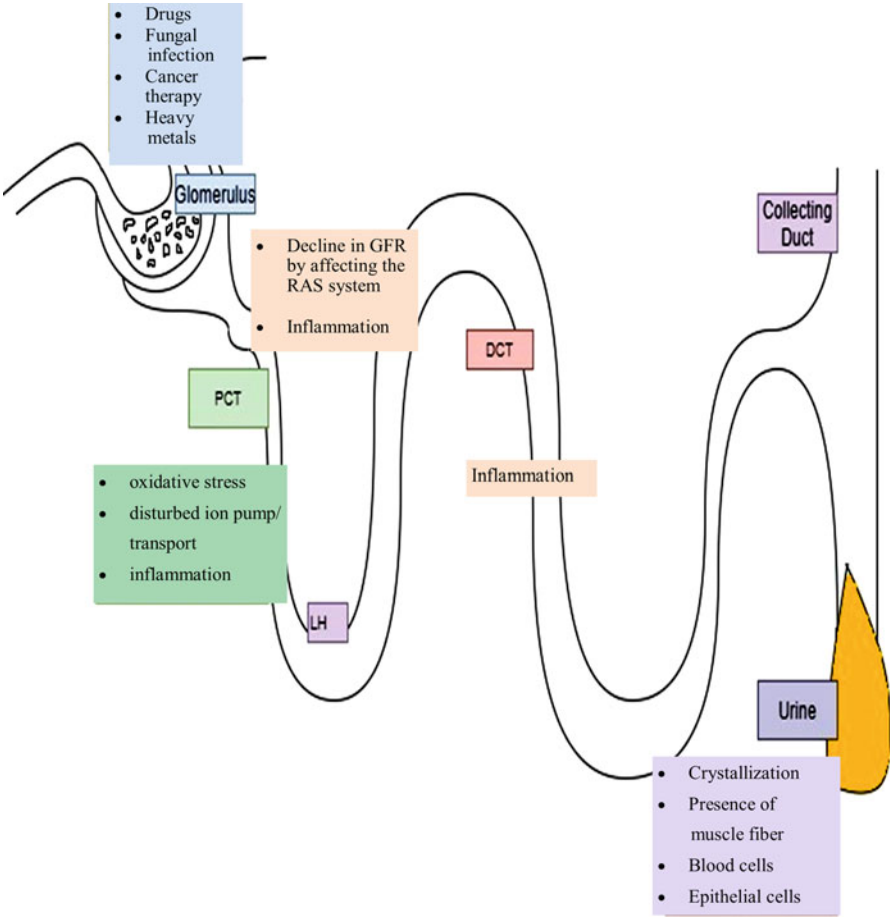


Fig. 2 Types of alterations observed in the nephron. This figure depicts different alterations that impact different parts of nephron.

epithelial cells of renal tubule (Pisoni et al. 2001). Anti-platelet medications such as cyclosporin, clopidogrel, mitomycin-C, and quinine can induce thrombotic microangiopathy (Pisoni et al. 2001; Manor et al. 2004).

Nephrotoxicity Biomarkers

As per definition given by the National Institute of Health (NIH), a Biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention." It allows the determination of interaction of exogenous toxicant with the tissues and sometimes infer mechanism that is affected while injury progression. Biomarkers are characterized with noninvasive nature, economical and

supplementing instant results. It allows detection from uncomplicated sources such as blood and urine. It possesses high specificity and sensitivity, allows early diagnosis, predicts prognosis, and is biologically plausible. Nephrotoxicity biomarkers are advancing with the knowledge (Refer to Table 2) and thus can be classified accordingly as conventional and novel biomarkers.

Conventional Biomarkers

These are traditional (non-invasive) and reliable markers used in the assessment of the nephron damage (Kim and Moon 2012). These utilize simple process such as

Table 2 Importance of biomarkers. This table summarizes all the biomarkers of drug-induced nephrotoxicity

Biomarker	Nature of biomarker	Affected organ/tissue/segment	Presence in serum/plasma/urine
Creatinine	Metabolism product	–	Serum
GFR	Amount of creatinine clearance	–	Urine
Blood urea nitrogen (BUN)	Metabolism product of protein	–	Urine
Albuminuria	Protein	Glomerulus and PCT	Urine
Cystatin C	Protein molecule	Glomerulus and PCT	
U-NGAL	Polypeptide	PCT, LH, and DCT	Urine/plasma
ILs	Cytokine	PCT and DCT	Urine
KIM 1	Epithelial protein	PCT	Urine
L-FABP	Transmembrane protein	PCT and DCT	Urine
IGFB7 × TIMP2		PCT	Urine
Calprotectin	Inflammatory protein	Inflammation	Urine/serum
U-AG	Protein hormone	PCT	Urine
U-miRNA	Micro RNA	Glomerulus, PCT, LH, DCT	Urine
Clusterin	Glycoprotein	PCT and DCT	Urine
βmacroglobulin	Plasma protein	Glomerulus and PCT	Urine
Osteopontin	Protein	PCT, LH, and DCT	Urine
Type IV collagen	Structural protein	Glomerulus	Urine
Cystatin C	Protease inhibitor	Glomerulus and PCT	Serum/urine
Brain natriuretic peptide	Secretory peptide	Damaged blood walls	Serum
Soluble ST2	Interleukin receptor protein	Fibrosis	Serum/plasma
Galectin-3	Protein	Present on macrophages and epithelial cells	Serum/urine

sedimentation of by-product or metabolic product or histopathological investigation. These may include:

- Determination of physiological/metabolic products such as creatinine in urine/plasma and urea in serum
- Measurement of glomerular filtration rate
- Evaluation of level of blood urea nitrogen
- Estimation of concentration of albumin in the urine, i.e., albuminuria
- Urine microscopy or microscopy of urine sediment

Creatinine

Level of creatinine is an early indicator of renal health. For disease diagnostics, only assessment of creatinine concentration in plasma cannot justify the condition, so we need to investigate the levels of GFR and blood urea nitrogen (BUN) as well.

Citrulline is a non-essential amino acid which is produced in the liver or small intestine. In tissues, protein (glycine, methionine) undergoes metabolization during urea cycle and produces citrulline. It is then transported via blood and taken up by the kidney, where it gets converted into arginine (L) appropriately by the PCT of the nephron. The arginine synthesized yields L-ornithine and guanidinoacetic acid (GAA) catalyzed by L-arginine and glycine amidinotransferase (AGAT). Simultaneously, GAA is methylated by *S*-adenosyl-L-methionine:*N*-guanidinoacetate methyltransferase (GAMT) to produce creatine (113 Da protein). Through the spontaneous, non-enzymatic, and equilibrium reversible process, creatine is changed to creatinine in a pH- and temperature-dependent manner. Creatine is observed at high pH and low temperature, while creatinine at vice-versa condition. Creatinine is the final product of creatine and is eliminated from the kidney. Abundance of creatinine in plasma directs improper functioning or damaged kidney (refer to Fig. 3).

Normal creatinine levels range from 0.9 to 1.3 mg/dL in men and 0.6 to 1.1 mg/dL in women from 18 to 60 years old. Creatinine is generally reported to be on higher side in person with muscular body as this component is said to be proportional to body mass, i.e., waste/product made by body muscles. Additionally, it has been reported to be influenced by extra renal factors such as age, sex, diet, medications, amputations, and hydration status. Laboratory methods to measure the amount of creatinine involve Jaffe, compensated Jaffe, enzymatic liquid, and dry chemistry methods. More recent and advanced technique includes isotope dilution-mass spectrometry (ID-GC/MS), which is considered as gold standard method (Hoste et al. 2015).

Alteration in creatinine levels by drugs: Kidney is accidentally exposed/targeted by many drugs that are in regular medication. Renal function is affected by interaction with the drugs. These drugs or their metabolites tend to interact either during glomerular filtration or/and by virtue of their charge that enable them to enter tubule epithelial cells via pinocytosis or endocytosis (Perazella 2009). Certain drugs (adefovir; fexofenadine) can be transported via peritubular capillaries and gain access to renal tubular epithelial cells at the basolateral surface. Regularly, from

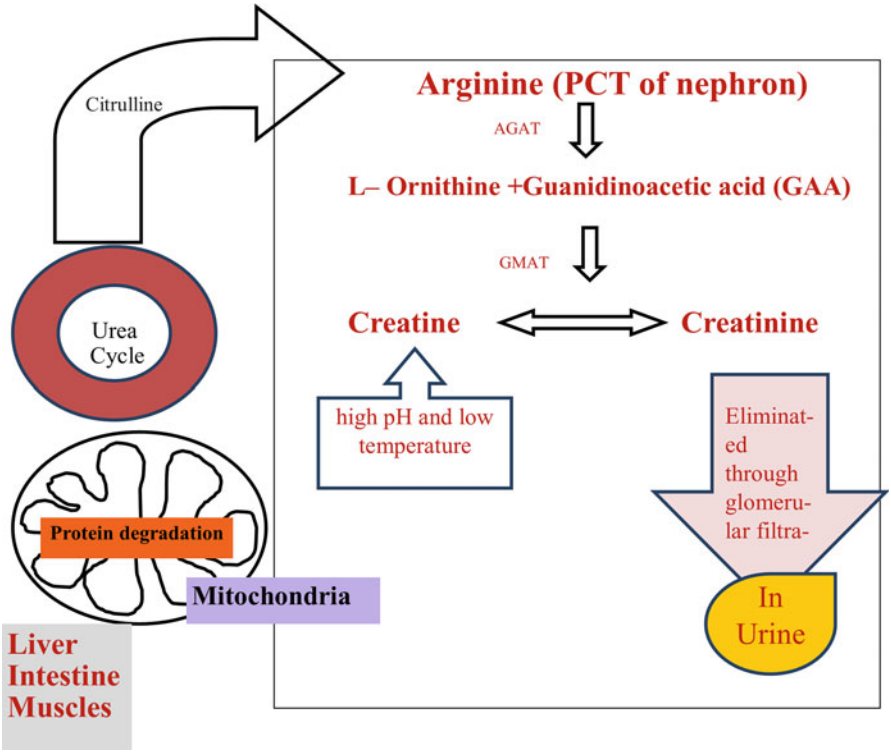


Fig. 3 Illustrating the metabolism and elimination of creatinine. This figure illustrates the metabolism of creatinine

there, these are taken up by organic anion and cation transporters (OATs and OCTs, respectively) and eventually effluxes into tubular lumens.

Consumption of certain drugs influences the creatinine concentration in urine either by virtue of parenchymal injury or altered renal hemodynamic or through dehydration of pre-renal fluid (Andreev et al. 1999). Another important group of drugs, corticosteroid, has been found to influence the creatinine levels in association with GFR. The elevated concentration of creatinine can be dedicated to the catabolism of protein and muscle tissue in response to the steroids (Beaufrere et al. 1989). Calcitriol (vitamin D3) also reported to induce plasma membrane injury or phospholipid loss in patients with acute renal failure (ARF) (Zager 1997).

However, drugs such as sulfamethoxazole, cimetidine (Tagamet), ranitidine (Zantac), cephalosporin, trimethoprim, pyrimethamine, phenacemide, salicylates, fenofibrate, and active vitamin D metabolites could also produce false alarming levels of creatinine without any decline in associated parameter, i.e., glomerular filtration rate (Andreev et al. 1999). Cimetidine (Tagamet) used in treating stomach

ulcers and preventing backflow of the gastric juice has been reported to enhance the creatinine concentration in plasma. This can be accredited to its higher affinity for the carrier protein compared to creatinine in proximal convoluted tubule, which leads to the reduced elimination of creatinine from the body. Trimethoprim, antibiotic in bacterial infections (bladder, kidney, and ear infections), and Pyrimethamine have shown congruent effect by competitively binding to transport carrier protein in PCT, thereby reducing creatinine excretion and accumulating it in plasma (Bugge 1996; Opravil et al. 1993).

Glomerular Filtration Rate (GFR)

GFR is among the important marker of renal health since late twentieth century. Considering the presence of creatine in muscle with subsequent nonenzymatic production into creatinine and its filtration through glomerulus marks essential biomarker of kidney health. In healthy females normal creatinine clearance is 88–128 mL/min and in healthy males it is 97–137 mL/min in a day. This can be noted and verified for the proper functioning of the kidney. Alteration in the amount of urine output can even precede biochemical changes.

In healthy individuals, glomerular filtration rate is around 120 mL/min/1.73 m^2, which is the amount of plasma filtered through the glomerulus. GFR is controlled by self-regulation of glomerular pressure mediated through prostaglandins (blood vessel – constriction and dilation) and enzyme such as angiotensin II. Certain drugs such as NSAIDS possess anti-prostaglandin activity, which interferes with the activity and influence GFR. Similarly, drugs such as angiotensin-converting enzyme (ACE) and angiotensin receptor blocker (ARB) can function against angiotensin and affect the rate (Palmer 2002).

Blood Urea Nitrogen (BUN)

Blood urea nitrogen (BUN) level is another important kidney function test. Urea is the basic metabolic by-product of N-containing protein by the liver. It is generally filtered by kidney, but its presence in blood determines the improper filtration mechanism. Normal urea concentration in human blood is 6–20 mg/dL. Detectable higher amount of urea is found in blood after 24–48 h of administration of AKI. The BUN-to-creatinine ratio generally provides more precise information about kidney function and its possible underlying cause compared with creatinine level alone.

Albuminuria/Proteinuria

It is the presence of high amount of albumin or protein in the urine due to improper functioning (damaged glomerulus or tubules) of renal system. Elevated secretion of albumin is observed in response to various agrochemicals such as imidacloprid, paraquat, and glyphosate (Roberts et al. 2010; Mohamed et al. 2017). Albumin is measured in terms of the urinary albumin-creatinine ratio (ACR) which stands for 30 mg/g (normal); values higher than this represents renal malfunctioning (NIDDK 2016). Presence of albumin is determined by dipstick test and ACR ratio.

Urine Sediment Microscopy

It is one of the rapid and sensitive techniques utilizing the identification of cell sediment present in the urine. If expertized, it can be used for the identification of epithelial cells, erythrocytes, leucocytes, lipid droplets, crystals, bacteria, and virus (Andersen et al. 2014).

Novel Biomarkers

The primary rationale for the development of novel biomarkers of kidney was the early and more accurate diagnosis and/or prognosis of kidney related diseases. In case of kidney injury the rise in the level of conventional biomarkers is relatively late. Thereby preventing early diagnosis. With the development of novel markers, early identification allowed for injury-specific interventions. This has been particularly useful in acute kidney injury (AKI) where sudden loss of kidney function (ARF) could be evident. Effective risk stratification and treatment could be planned in cases of AKI following cardiac procedure and other complications. There is a need of specific and comparable markers for early diagnosis of the nephron damage and toxicity. Other parameters suggesting the need are

- Creatinine-based diagnosis occurs almost after 50% kidney damage (Dharnidharka et al. 2002).
- Evolvement of a marker that will be independent of other non-kidney factors, unlike creatinine dependent on protein metabolization, muscle mass, and diet.
- It must prevent misleading diagnosis, as apparent in cases of tumor growth, where chemotherapy and loss of muscles leads to overproduction of creatinine.

Thus, novel biomarkers have several potential roles in nephrology like:

- More precise diagnosis of AKI, relatively initially in the development of the disease itself.
- Development of better relationship with GFR in CKD patients.
- Identification of the anatomic (glomerulus, tubules, interstitial cells, or vascular) location of injury in AKI.
- Identification of specific markers as for structural injury, damage and repair, and tubular function.
- Determination of disease-causing agents (drugs) of AKI and CKD.
- Regular monitoring of the effectiveness of interventions and therapy.
- Availability of information relating to the prognosis of acute kidney infection and chronic kidney disease.

Due to recent developments the nephrotoxicity biomarkers that have been developed are

- Neutrophil gelatinase-associated lipocalin (NGAL) in urine
- Interleukins-18 and 16
- Kidney injury molecule 1 (KIM-1)
- Liver-type fatty acid-binding protein (L-FABP)
- Insulin-like growth factor-binding protein 7 (IGFBP7) and tissue inhibitor of metalloproteinases-2 (TIMP-2)
- Calprotectin
- Urinary angiotensinogen
- Urinary microRNA
- Cystatin C
- Clusterin

Neutrophil Gelatinase-Associated Lipocalin (NGAL)

It is a polypeptide (25 kD) belonging to a lipocalin family. It is extensively expressed in cells (such as neutrophil, hepatocytes, renal tubular epithelium during inflammation) and functions as a growth and differentiation factor. In renal epithelium, it has role in iron trafficking (Schmidt-Ott et al. 2007). Principal sites for NGAL production are the thick portions of ascending limb of Henle loop and most abundant intercalated cells of the collecting duct of the nephron (Schmidt-Ott et al. 2007).

Both urine and plasma show prevalence of NGAL protein in as early as 3 h (peak 6–12 h) in response to inflammation, ischemia, and injury (Mishra et al. 2003). NGAL serves as a reliable marker in case of ischemia, sepsis, or posttransplantation acute kidney injury, in pediatric patients of AKI, and decompensated cirrhosis (Zhou 2017; Udgirkar et al. 2020).

Interleukins

Interleukins are the pro-inflammatory cytokines released by immune cells in order to recruit, proliferate, and differentiate other immune cells as a function of immune reaction. IL-18 is produced by infiltrating macrophages or monocytes or damaged tubular epithelium cells and serves as biomarker for the parenchymal tissue injury, renal tubule-interstitial fibrosis in case of CKD (Liang et al. 2007). Its presence in urine makes it a diagnostic marker. (Parikh et al. 2004).

Interleukin 6 (IL-6) is a multifunction cytokine with role in immunogenic reaction accompanied to hematopoiesis and metabolism. It can be produced by a variety of renal cells such as podocytes and tubular epithelial cells TECs (Su et al. 2017). IL-6 in serum serves as important diagnostic marker in end-stage renal disease (ESRD) patients, chronic renal failure CKI, and AKI (Su et al. 2017). But compared to NGAL, these interleukins (IL-18 and 6) provide a generalized diagnostic, especially in aged patients (Gan and Zhou 2018). Moreover, these do not display strong cutoff value and thus conflict the findings (Lin et al. 2015).

Kidney Injury Molecule 1 (KIM 1)

It is type 1, transmembrane protein (comprising of immunoglobulin and mucin domain) apparent on the apical membrane of PCT. It is a molecule that is produced

in stress and injury in tubules following ischemia and AKI (Khreba et al. 2019). KIM 1 protein possesses phosphatidylserine receptors that act for directing apoptotic cells to lysosomes and signal them "eat me up" (Savill and Fadok 2000).

This molecule possesses high organ specificity and sensitivity as a biomarker, being evident only in case of PCT injury and remains until fully recovered. Its apical domain sustains on PCT while stable ectodomain (90 kD) is released in the urine (Bonventre 2008). It is included in the list of nephrotoxicity marker of FDA and EMEA in case of renal insult by various toxicants such as drugs and agrochemicals (FDA 2008; Zou et al. 2017). Presence of KIM 1 in urine demarcates the acute tubular necrosis (ATN) (Han et al. 2002).

Liver-Type Fatty Acid-Binding Protein (L-FABP)

These are small proteins (14–15 kDa) found inside cytoplasm of human PCT. These bind to fatty acids and causes its metabolization by oxidation. It therefore serves as endogenous antioxidant. Quantity of the protein is estimated by the ELISA. Presence of these protein has been observed in the cisplatin-induced toxicity and ischemia reperfusion injury (Negishi et al. 2007).

Calprotectin

It is a pro-inflammatory mediator protein belonging to the innate immune system. It is released by the neutrophil at the site of inflammation. Urinary calprotectin is helpful in diagnosis of adult as well as pediatric AKI (Westhoff et al. 2017). Fecal calprotectin serves as biomarker for the gastrointestinal disease as well (Andreasson et al. 2011).

Brain Natriuretic Peptide (BNP)

BNP is produced by the heart cells and walls of blood vessels. It functions to maintain cardiac-renal homeostasis by inhibiting the sodium reabsorption in the PCT and DCT, along with improving the GFR and renal plasma flow. These serve as biomarker for the cardiac failure as well as for the AKI (Akgul et al. 2014). Elevated BNP serum levels are observed in case of CKD patients due to renal impairment (non-clearance of BNP), tubular necrosis, and inhibited neprilysin.

Soluble ST2

It is a member of interleukin receptor family and stands for soluble suppression of tumorigenicity-2. It is a novel marker of heart failure and cardiovascular stress (Kim and Park 2022). It acts as predictive of AKI and CKD in patients associated with myocardial infarction (Tung et al. 2015) and end-stage renal disease ESRD at higher values, i.e., >1500 pmol/L in serum/plasma (Homsak and Gruson 2020).

Galectin-3

Galectin-3 is a beta-galactoside binding lectin and a multipurpose protein functioning for growth, differentiation, and apoptosis and expressed on the surface of the macrophages and epithelial and endothelial cells. It is associated with the process of

fibrosis and renal failure, thus, if found in the cytoplasm, serum, and urine, indicates progression of the disease and also serves as biomarker of cardiac disease, lung fibrosis, cancer, and renal dysfunction (Ho et al. 2012; Tang et al. 2011). It can serve as predictive indicator for both AKI, CKD, and diabetic nephropathy (DN) (Sun and Lerman 2019). Its elevated concentration directs toward the progression from microalbuminuria and macroalbuminuria indicative of glomerular inflammation (Kikuchi et al. 2004).

IGFBP7 x TIMP2

In an initiative for early diagnosis of AKI, "NephroCheck" has been introduced on September 5, 2014 by FDA (US-FDA 2014). It talks about two independent biomarkers as the arithmetic product of TIMP2 × IGFBP7. Tissue metalloproteinase inhibitor 2 (TIMP 2) is a gene encoding for protein (24 kD). It acts for the regulation of metastasis and cell repair. However, IGFBP7 are insulin-like growth factor-binding proteins (34 kD) expressed in the tumor cell. Their upregulation in the urine can be conferred to each exert cell cycle inhibitory effects and thus act as "G1 cell cycle arrest biomarkers" (Johnson and Zager 2018).

Urinary TIMP2 and IGFBP7 protein concentration was found to be significantly higher (ten-fold) in as early as 4 h of renal injury. This elevation can be accounted for the damaged brush border lining, inhibition of reabsorption by the tubules, and increased filtration through glomerulus, marking it as the appropriate maker for renal injury (Johnson and Zager 2018).

As said "with position comes the responsibility" so being its high superiority it has certain limitations. It needs confirmation of the outcome by the serum creatinine levels and must not be performed in patients under age 21, with minor surgeries, and in patients with kidney disease (Vijayan et al. 2016). Value of >0.3 for the TIMP2 × IGFBP7 indicates moderate to severe AKI for 12 h, while lower value represents lower risk (Vijayan et al. 2016; NephroCheck 2014) and value of >2 is associated with high risk (US-FDA 2014).

Urinary Angiotensinogen (UAG)

Renin-angiotensin system (RAAS) acts on the renal tissue for the regulation of water balance and blood pressure (Ferrao et al. 2014). It also promotes fibrosis mediated by inflammation (Kobori et al. 2007). Angiotensinogen and angiotensin II are expressed in the tubular cells upon injury. AKI patients have also reported elevated level of this angiotensinogen in urine, representing its progression to AKD (Cui et al. 2018). In order to have check over the progression, there is a need to determine the health of the tissue. Histopathological assay can be used for determination of structural integrity of the renal tissue. Here, determination of urinary angiotensinogen (UAG) can serve as reliable source indicator of structural integrity. It has been observed that in patient following treatment of AKI, higher UAG reflects the process of fibrosis and repair going on. While, with structural restoration, its level declines (Cui et al. 2018). It is a novel and early biomarker of activation of the renin-angiotensin system in type 1 diabetes.

Urinary MicroRNA

miRNA is a small (20 nt) oligonucleotide RNA that acts for various mechanism post transcription. These exhibit differential expression on the tissue, and qRT-PCR is the best suited technique available for the detection of miRNA and its profiling. Along with liquid waste, these miRNAs are flushed out in urine, after filtration from glomerulus or are released by the renal cells. miRNA 21 is dually associated with AKI, as it functions to inhibit apoptosis and inflammation on one hand and promotes fibrosis and help in cellular repair on the other hand (Sun and Lerman 2019). Along with its suitability as urinary biomarker, it can also evidence ischemia-reperfusion injury (IRI) (Saikumar et al. 2012). miRNA-24, miRNA-30c-5p, and miRNA-192-5p expression has been found related with the IRI-induced AKI (Zou et al. 2017). Expressed miRNA-494 is related to apoptosis and inflammation in the tubule (Lan et al. 2012). Studies have reported miRNA 15, miRNA-126, and hsa-miRNA-770 as urinary markers associated with the CKD and diabetic nephropathy (Park et al. 2018). Recently, Shihana et al. (2020) demonstrated urinary miRNA, viz. miR-30a-3p, miR-30a-5p, miR-92a, and miR-204, with around >17-fold change at $p < 0.0001$ exposed to snake envenoming, and intoxication with paraquat and oxalic acid. Other, miRNA involved in nephrotoxicity assessment are miR-191 and miR-30a-5p20 (Kanki et al. 2014); and miR-423 (Pavkovic et al. 2016); miR-210 (Neal et al. 2011), miR-16, miR-21, miR-155, and miR-638 in CKD patients (Neal et al. 2011).

Drug-induced miRNA has been noticed in nephrotoxicity (Chorley et al. 2021). It was depicted from the experimental studies that there is a nephron segment wise expression of miR-23a-3p in glomerulus, miR-192-5p in PCT; miRs-221-3p, -22-3p, and -210-3p in thick ascending limb, and in collecting duct. Similarly, Wang et al. (2012) conferred miR-10a and miR-30d as novel markers in renal injury specifically identifying the focal segmental glomerulosclerosis in mouse exposed to renal ischemia-reperfusion and streptozotocin. Jeon et al. (2020) elucidated the gentamicin-induced expression of let-7g-5p, miR-21-3p, 26b-3p, 192-5p, and 378a-3p in the urine of rats suffering from necrosis in PCT.

β2-Microglobulin (β2M)

It is a 100-amino-acid-long, low-molecular-weight (11 kDa) protein associated with the MHC molecules. These plasma proteins (β2-microglobulin, α1-microglobulin) are freely filtered by the glomerulus. Its presence in the urine could be marked to the inability of the association with MHC and its subsequent release as filtrate or dysfunction of urinary system by inhibiting megalin-mediated endocytosis in the renal tubules. Gentamycin-induced nephrotoxicity caused inhibited transport proteins thereby preventing the endocytosis of the protein (Gautier et al. 2014).

Cystatin C (Cys C)

Cys C is a cysteine protease inhibitor which is synthesized by all nucleated cells of the body and distributed in body fluids (Onopiuk et al. 2015). It is generously filtered

through the glomerulus and metabolized in PCT, but not released in the serum due to endocytic reabsorption in the PCT (Onopiuk et al. 2015). Cys C levels remain constant and unaltered by metabolism (renal conditions, rate of protein catabolism, dietetic factors) or with factors like age or muscle mass unlike creatinine. Thus, it serves as endogenous marker of GFR at early stages of both acute and chronic kidney disorder. Its presence in the serum is entirely dependent on kidney function.

Certain anti-microbial agents such as Colistin have been reported to induce nephrotoxicity. Evident cellular swelling and cell lysis is due to the influx of anions, cations, and water due to the increased tubular epithelium permeability. Comparative studies have illustrated higher accuracy of Cys C compared to serum creatinine in various animal models. In patients with chronic kidney disease, Cys C seems to be a reliable marker for the early diagnosis and prognosis of contrast-induced acute kidney injury.

Clusterin

It is a small 80 kDa glycoprotein secreted by the urinary tract cells. These are expressed in case of apoptosis and stress (Park et al. 2014). In case of renal injury or dysfunctional PCT, these are observed in the urine. Drugs such as gentamycin and everninomicin caused tubular nephrotoxicity, which was evidenced by the higher concentration of clusterin protein in urine output (Davis et al. 2004). Elevated amount of clusterin was also observed in cisplastin-induced nephrotoxicity and, as confirmed by the immunohistochemistry, suggested tubular necrosis and cell detachment in case of AKI (Aslan et al. 2018).

Collagen

Certain proteins, especially low-molecular-weight peptides that are specific to certain function or structural components, if present in urine sample can serve as marker for the damage (Rouse et al. 2012). Abundance of proteins, such as forming structural component of collagen, and breakdown product of these can be determined in a sample through capillary electrophoresis–mass spectrometry or tandem mass spectrophotometry. Presence of fragments of the collagen chains, i.e., alpha-1 (I), alpha-1 (III), and alpha-2 (I), inferred collagen damage developed by the gentamicin. Corresponding upregulated metalloproteinase (MMP 2 and MMP 9) observed suggested the presence of enzyme responsible for collagen degradation (Basile et al. 2001; Forbes et al. 2000). Basement membrane of glomerulus is made by type IV collagen, and its filtration through glomerulus is specific to damaged glomerulus (Donovan et al. 1994; Nerlich et al. 1994).

Osteopontin

These are important moiety, acting for activation of T lymphocytes, a bone protein, and a tubular glycoprotein secreted in both phosphorylated and non-phosphorylated forms. It is bone and epithelial protein which marks the injury especially glomerulonephritis, if found in urine. Verstrepen et al. (2001) observed

drug gentamicin-induced osteopontin gene and protein expression in PCT and DCT suggesting ARF.

Symptoms of Nephrotoxicity

Most common symptoms of AKI may include:

- Fatigue
- Irregular heartbeat
- Shortness of breath
- Confusion
- Decreased urine output
- Nausea
- Weakness
- Pressure in chest and pain
- Retention of fluid and swelling in ankles, legs, and feet
- Seizures or coma in severe cases

Diagnostic Tests for Nephrotoxicity

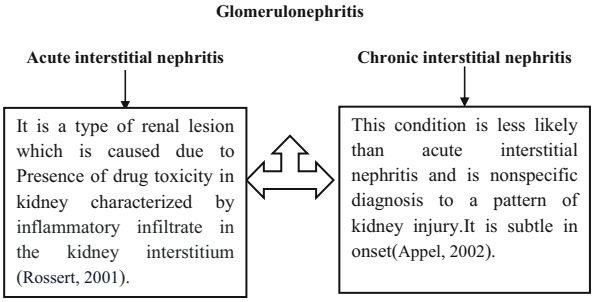
There are several clinical laboratories that perform kidney function tests and blood and urine tests to check the proper functions of kidney.

- Blood sample is taken to check whether the kidneys are filtering the blood properly or not. Also blood sample test is used to check the presence of nitrogenous product, i.e., urea. Kidney plays a very important role in filtration of urea from the blood. The presence of excess of nitrogen depicts the abnormal function of kidney.
- Serum from the blood is used to examine the level of creatinine.
- Urine sample is taken to check the albumin content in the urine. When the kidneys get damaged, albumin can pass into the urine.
- A creatinine clearance test measures the amount of creatinine in a blood sample taken from your arm and in a sample of all the urine you produce during a 24-h period. Creatinine develops as a result of the breakdown of creatine, a chemical that helps provide the muscles with energy.
- Physical examination of urine assesses the chemical and microscopic examination which involves clarity and color of urine. Darker urine informs about dehydration in body while normal urine is straw in color. The presence of redness in urine may specify the porphyria or hematuria. Presence of glucose in urine informs about diabetes mellitus.
- The microscopic analysis involves a wet-prep analysis of urine to assess the presence of cells, casts, and crystals as well as microorganisms.

Disorders Related to Nephrotoxicity

Glomerulonephritis

Glomerulonephritis (GN) is an inflammatory condition of glomerulus caused primarily by immune mechanisms and is often associated with the presence of protein in urine (Perazella 2005). On the basis of causes, GN is categorized into acute and chronic condition.



Interference in Intra-renal Renin-Angiotensin System

The kidney plays a very important role in maintaining autoregulation by modulating the tone of efferent and afferent arteries to maintain GFR and urine output. During severe dehydration, intraglomerular hemodynamics and autoregulation become impaired and there is more marked renal vasoconstriction which decreases the GFR (Katherine et al. 2011). Drugs having anti-inflammatory activity or those with antiangiotensin-II activity inhibitors or receptor blockers interferes with autoregulation of kidney and decreases the GFR (Palmer 2002; Schoolwerth et al. 2001) leading to renal impairment.

Renal Tubule Acidosis

By exposure to certain drugs and chemicals, kidneys suffer a number of tubular dysfunction syndromes, i.e., proximal renal tubular acidosis or full-blown Fanconi syndrome (Schetz et al. 2005; Izzedine et al. 2005; Markowitz and Perazella 2005). Sometimes nephrogenic diabetes insipidus occurs by toxicity of heavy metals such as lithium, cadmium, and acyclic nucleotide phosphonates (Yu et al. 2004).

Chronic Kidney Disease (CKD)

CKD is the longstanding disease of the kidneys that leads to complete renal failure. Combination of different types of drugs such as aristolochic acid-containing herbal

remedies, analgesics, and other complementary product are very harmful for kidney (Isnard et al. 2004; Falagas and Kasiakou 2006).

Preventing Drug-Induced Impairment

It is generally suggested that preventive measures should be taken to avoid nephrotoxicity. General methods include the dosage of non-nephrotoxic drugs which have high efficacy and least risk factors. A proper care should be taken into account for various factors like assessment of baseline renal physiological functions and the dose of medications required. As suggested by the National Kidney Foundation, there are different formulations for calculation of renal factors for young ones and adults. The drugs which are excreted renally do not need any dosage adjustment and changes until it affects the creatinine levels in the body. It is further suggested that enough hydration is vital and important to retain renal perfusion and evade any sort of drug-induced renal damage and impairment. However, at present, there is no agreement and accord on the optimal level and volume of fluid required to have optimum level of renal perfusion. While some researchers suggest that a systematic concept of computerized physician order entry and medical result system may decrease the risk involved in exposing patients at risk to nephrotoxins, but much more research is required to have some concrete decision.

The efficacy and potential of different drugs vary according to the infection and the complexity involved. Though efforts and research are focused to minimize the side effects of the drug-induced toxicity, still the full potential is yet to be explored and the path is being paved by findings of different researchers. Typically, drug-induced nephrotoxicity seems to have been more likely to patients which depict certain sort of clinical situations. Therefore, the research and study should be designed in such a way that it should explore the drug-related risk factors and should look for preemptive measures and early intervention of induced toxicity. The preventive strategies should target and employ those methods which involve the study in relation to prescribing and monitoring of potential nephrotoxins in at-risk patients, and, if possible, risk factors should be corrected before drugs associated with nephrotoxicity are prescribed.

Applications to Prognosis, Other Diseases, or Conditions

In this chapter, conventional and novel biomarkers of kidney disease are discussed. As these conventional biomarkers of kidney disease develop/raise relatively late in the injury process or after actual injury has incurred. Thus, preventing early diagnosis and leads to propagation of the injury and disease. Traditionally used biomarkers of drug-induced nephrotoxicity are glomerular filtration rate, albumin, creatinine, blood urea nitrogen. There is a great need of novel nephrotoxicity biomarkers for early diagnosis as it can prevent the progression into renal failure. Some novel biomarkers studied by different scientists like Neutrophil Gelatinase-

Associated Lipocalin, Kidney Injury Molecule 1, Liver-Type Fatty Acid-Binding Protein, Calprotectin, Brain Natriuretic Peptide, Soluble ST2, Galectin-3, Urinary microRNA, β2-Microglobulin, Cystatin C, Clusterin have been reviewed which can be used to access the nephrotoxicity. In this study, we have listed different drugs available in market that can be potentially toxic to the kidneys. We have also described different mechanisms involved in drug-induced nephrotoxicity. Chronic drug-induced nephrotoxicity leads to glomerulonephritis, disturbs intrarenal renin-angiotensin system, renal tubule acidosis, or can cause chronic kidney disease, or even renal failure in some cases. It is generally suggested that preventive measures like dosage regulation, hydration therapy, use of some supplements to reduce toxicity, etc. should be taken to avoid nephrotoxicity. Studying the mechanisms of drug-induced nephrotoxicity and exploring these biomarkers can provide us with very useful information that can further help us in the development of safe drugs.

Mini-Dictionary of Terms

- **Acidosis:** Abnormally high acidity of the blood and body tissues caused by a deficiency of bicarbonates or an excess of acids other than carbonic acid. It can result from any of various acute or chronic disorders or from the ingestion of certain drugs and toxins. Also called *metabolic acidosis*.
- **Acute kidney injury (AKI):** An abrupt loss of kidney function.
- **Agrochemicals:** Chemicals that are used to kill plants, animals, and other pests in agriculture.
- **Albumin:** Water-soluble proteins produced by liver and found in serum.
- **Albuminuria:** The presence of albumin in the urine, sometimes indicating kidney disease.
- **Alkalosis:** Abnormally high alkalinity of the blood and body tissues caused by an excess of bicarbonates, as from an increase in alkali intake, or by or a deficiency of acids other than carbonic acid, as from vomiting. Also called *metabolic alkalosis*.
- **Amidinotransferase:** A family of enzymes involving transfer of amidino[–C(=NH)NH2] group in a reaction.
- **Amputations:** To cut off/remove a body part. It can be natural, accidental, or surgical.
- **Analgesics:** A medication that reduces or eliminates pain.
- **Angiotensin:** A peptide hormone that causes constriction of blood vessels and helps in maintaining blood pressure.
- **Angiotensinogen:** It is α_2-globulin precursor of angiotensin. It has physiological importance in regulation of blood pressure.
- **Apoptosis:** A natural process of self-destruction of the cells by the release of hydrolases from lysosomes or simply called *programmed cell death*.
- **Autoimmune disorders:** Diseases characterized by abnormal functioning of the immune system that causes immune system to produce antibodies against our own tissues.

- **Basolateral:** Basal and lateral; specifically used to refer to one of the two major cytologic divisions.
- **Beta-galactoside:** A glycoside that yields energy by break down of lactose to galactose and glucose on hydrolysis. Also called lactase.
- **Biomarker:** A distinctive biological or biologically derived indicator of a process, event, or condition.
- **Carbonic anhydrase:** An enzyme that accelerates the reaction between carbon dioxide and water to form carbonic acid in the red blood cells.
- **Catabolism:** The breakdown of complex body molecules to simpler forms, as when muscle protein breaks down to amino acids or fats to glycerol and fatty acids.
- **Cirrhosis:** A chronic degenerative disease in which normal liver cells are damaged and are then replaced by scar tissue.
- **Clusterin**: Heterodimeric highly conserved secreted glycoprotein, which is expressed in a wide variety of tissues and found in all human fluids and associated with cellular debris and apoptosis.
- **Co-morbidity:** A concomitant but unrelated pathologic or disease process; usually used in epidemiology to indicate the coexistence of two or more disease processes. For example, diabetes and heart disease.
- **Cystatin C:** It is a low-molecular-weight protein (13 kDa) that is produced by all nucleated cells and acts to inhibit cysteine proteases. Serve as biomarker of kidneys health.
- **Cytokine**: A low-molecular-weight protein that influences proliferation, differentiation, and function of immune system cells. For example, interleukins and interferons.
- **Cytotoxicity**: The degree to which an agent has specific destructive action or toxicity on cells.
- **Dipstick test:** A thin, plastic stick with strips of chemicals on it – is placed in the urine to detect abnormalities. The chemical strips change color if certain substances are present or if their levels are above normal.
- **Drug:** Any composition that can be used to treat, prevent, diagnose, and relieve symptoms of an infection or disease.
- **Electrophoresis:** It is a laboratory technique used to separate DNA, RNA, or protein molecules based on their size and electrical charge.
- **Endocytosis:** The process of internalization of material into the cell from an outside environment by invagination of the plasma membrane.
- **Fibrilization:** An act or process of forming fibers or fibrils.
- **Fibrosis:** The development of fibrous connective tissue as a reparative response to injury or damage.
- **Galectin:** A class of protein that binds specifically to the beta-galactoside sugars. It regulates cell division, apoptosis, and metastasis. Also serves as a potential biomarker of myocardial fibrosis in patients with heart failure.
- **Gastrointestinal**: Structures relating to the stomach and intestines.
- **Glomerulonephritis:** Renal disease characterized by inflammatory changes in glomeruli.

- **Glomerulosclerosis**: It is a deposition of hyaline deposits or scarring within the renal glomeruli.
- **Glycoprotein:** A class of conjugated proteins consisting of a compound of protein with a carbohydrate group.
- **Growth differentiation factor (GDP):** Protein from the TGF-β superfamily that has functions predominantly in the development of organism.
- **Guanidinoacetic acid:** Also known as glycocyamine is the natural precursor of creatine.
- **Hematopoiesis:** The process of growth and maturation of the blood cells and other formed blood elements in the bone marrow.
- **Hematuria:** The discharge of blood in the urine, making the urine either slightly blood-tinged, grossly bloody, or a smoky brown color.
- **Hemodynamics:** The study of the forces involved in the circulation of blood. It can also be referred as measure of cardiovascular function.
- **Histopathological:** The study of the anatomical changes in the tissue under microscopic.
- **Hyponatremia:** A condition of lower sodium than the normal level in the blood.
- **Immune reaction:** Antigen-antibody reaction indicating the specificity.
- **Immunohistochemistry:** It is a technique of diagnosis of specific antigens (protein) in tissues by the use of enzyme or dye-conjugated antibodies (as markers).
- **Inflammation:** A localized protective response elicited by injury or destruction of tissues, which serves to destroy, dilute, or wall off both the injurious agent and the injured tissue. It is characterized by the redness, swelling, edema, heat production, and loss of sensation.
- **Innate immune system:** The component of the immune system in animals that is genetically determined and is nonspecific. It acts as first line of defense.
- **Insulin-like growth factor-binding protein 7:** It is a secreted protein binding insulin-like growth factor 1 (IGF-1), insulin, vascular endothelial growth factor A (VEGFA), and activin A.
- **Interleukins (ILs):** These are a type of cytokine first thought to be expressed by leukocytes alone but have later been found to be produced by many other body cells. They play essential roles in the activation and differentiation of immune cells, as well as proliferation, maturation, migration, and adhesion.
- **Ischemia:** It is a condition of an insufficient supply of blood, indirectly oxygen to an organ, usually due to a blocked artery.
- **Isotope:** Types of atoms having the same atomic number as another but having a different atomic mass. For example, protium, deuterium and tritium isotopes of hydrogen.
- **Lectin:** A type of protein that binds to certain carbohydrates.
- **Lipocalin:** These are a large group of small extracellular proteins which have been associated with inflammation, transport of small hydrophobic ligands, such as steroids, lipids, and pheromones, as well as the synthesis of prostaglandins.
- **Liver-type fatty acid-binding protein (L-FABP)**: It is a 14 kDa protein found in the cytoplasm of human renal proximal tubules.

- **Lysosomes:** It is a membrane-bound cell organelle that contains digestive enzymes.
- **Macroalbuminuria:** It is a measure of urinary albumin-to-creatinine level and generally has values more than 300 mg/g Cr.
- **Macrophages:** These are white blood cell that helps eliminate foreign substances by engulfing foreign materials and initiating an immune response.
- **Major histocompatibility complex (MHC):** It is a large locus on vertebrate DNA containing a set of closely linked polymorphic genes that code for cell surface proteins essential for the adaptive immune system.
- **Mass spectrometry:** It is an analytical technique that is used to measure the mass-to-charge ratio of ions present in a sample.
- **Metabolites:** These are the intermediates and end products (e.g., amino acids, organic acid and bases, fatty acids, bile acids, lipids, and carbohydrates) of cellular regulatory processes.
- **Metalloproteinase:** It is a protein digesting (protease) enzyme whose catalytic mechanism involves metal such as Zn.
- **Metastasis:** In cancer, cells break away from the original (primary) tumor site and travel through the blood or lymph system and form a new tumor in other organs or tissues of the body.
- **Methyltransferase:** These are enzymes involved in transfer of a methyl group from S-adenosylmethionine (SAM) to their substrates.
- **Microangiopathy** (small vessel disease): A disease of the capillaries in which the capillary walls become so thick and weak that they bleed, leak protein, and blood flow becomes slower.
- **Microglobulin:** A protein that is found on the surface of nucleated cells and functions as part of the MHC system.
- **microRNA (miRNA):** It is a small single-stranded noncoding RNA molecule found in plants, animals, and some viruses that functions in RNA silencing and posttranscriptional regulation of gene expression.
- **Monocytes:** These are a type of leukocyte (white blood cell) and has a role in phagocytosis.
- **Myocardial infarction:** Commonly known as a heart attack, occurs when blood flow decreases or stops to a part of the heart, causing damage to the heart muscle.
- **Myocyte:** Structural unit of muscular tissue and organs throughout the body.
- **Necrosis:** Death of a circumscribed area of plant or animal tissue as a result of disease or injury.
- **Nephritis:** Condition in which the tissues in the kidney become inflamed and have problems filtering waste from the blood.
- **Neprilysin:** It is a zinc-dependent metalloprotease that cleaves peptides at the amino side of hydrophobic residues and inactivates several peptide hormones including glucagon, e.g., oxytocin.
- **Neutrophil gelatinase-associated lipocalin:** A polypeptide released by damaged nephron tubular cells in the setting of local inflammation.
- **Neutrophil:** A type of white blood cell that helps heal damaged tissues and resolve infections.

- ***N*-guanidinoacetate methyltransferase:** An enzyme that catalyzes the chemical reaction and is encoded by gene GAMT.
- **Oligonucleotide:** A compound comprised of about 3–20 nucleotides.
- **Osmolality:** The concentration of a solution expressed as the total number of solute particles per liter.
- **Osteopontin:** It is a highly phosphorylated protein that is a prominent component of the mineralized extracellular matrices of bones.
- **Pharmacological:** A branch of medicine, biology, and pharmaceutical sciences concerned with drug or medication action.
- **Phosphatidylserine receptors**: These are functionally different active receptors expressed by phagocytes.
- **Phospholipid:** Class of lipids whose molecule has a hydrophilic "head" containing a phosphate group, and two hydrophobic "tails" derived from fatty acids, joined by a glycerol molecule.
- **Pinocytosis:** A process by which liquid droplets are ingested by living cells.
- **Plasma:** It is the liquid (pale to yellow colored) portion of blood comprising salts, enzymes, and water.
- **Podocytes:** Cells in the Bowman's capsule in the kidneys that wrap around capillaries of the glomerulus.
- **Prognosis:** A doctor's judgment of the likely or expected development of a disease.
- **Prostaglandins:** A group of lipids made at sites of tissue damage or infection that are involved in dealing with injury and illness.
- **Protease:** An enzyme that catalyzes the breakdown of proteins into smaller polypeptides or single amino acids.
- **Proteinuria:** Increased levels of protein in the urine.
- **Renin-angiotensin system (RAAS):** It is an endocrine physiological system that regulates blood pressure, electrolyte balance, and systemic vascular resistance.
- **Reperfusion:** It is a tissue damage caused when blood supply returns to a tissue after a period of ischemia or lack of oxygen.
- **Rhabdomyolysis:** Serious syndrome due to a direct or indirect muscle injury. It results from the death of muscle fibers and release of their contents into the bloodstream.
- **Sepsis:** It is a serious medical condition caused by the body's response to an infection and start digesting own cells. It may develop to septic shock.
- **Syndrome:** Set of medical signs and symptoms which are correlated with each other and often associated with a particular disease or disorder.
- **Thrombosis:** It is the process of the formation of a blood clot, known as a thrombus, within a blood vessel.
- **Toxoplasmosis:** Toxoplasmosis is an infection caused by a parasite called *Toxoplasma gondii.*
- **Tumorigenicity:** It is the tendency for cultured cells to give rise to either benign or malignant growing tumors when infected to in immunologically nonresponsive animals.

- **Vasoconstrictor:** It is narrowing or constriction of the blood vessels by small muscles in their walls.

Key Facts of Drug-Induced Nephrotoxicity and Use of Biomarkers

- Kidney plays a very important role in regulation of osmolarity, maintenance of homeostasis, blood pressure regulation, and detoxification process.
- Nephrotoxicity is basically caused due to prescription of different combination of drugs which occurs more repeatedly in patients having history of other diseases like heart diseases, diabetes, chronic kidney disease, etc. as compared to other patients.
- Out of the total nephrotoxicity cases, approximately around 20% occurs by the use of different nonsteroidal anti-inflammatory drugs.
- These drugs basically alter the different regions of kidney and may cause tubular cell toxicity, inflammation, change in the hemodynamics of glomerulus, crystal nephropathy, rhabdomyolysis, thrombotic microangiopathy, and lastly chronic kidney disease.
- Several conventional (creatinine level, blood urea level, albuminuria, etc.) and novel (NGAL in urine, level of IL16 and IL18, KIM-1, TIMP-2, Urinary micro-RNA, Cystatin C, Clusterin, etc.) biomarkers are used to assess the drug nephrotoxicity.
- Several symptoms of nephrotoxicity include fatigue, irregular heartbeat, weakness, decreased urine output, etc. for early detection of nephrotoxicity.
- The safest measure we can prefer to protect nephrotoxicity is to avoid taking of different combinations of drugs or dosage of non-nephrotoxic drugs.

Summary Points

- Various factors which lead to nephrotoxicity includes anti-arthritic, antibiotics, antihistamines, antimicrobials, diuretics, metals, gastrointestinal drugs, sedatives, etc.
- Drug-induced nephrotoxicity adopts the mechanism which leads to changes in physiology of different renal parts and leads to hemodynamic changes, damage to cells and tissue, inflammation, obstruction of renal excretion, tubular cell toxicity, crystal nephropathy, rhabdomyolysis, and thrombotic microangiopathy.
- There are two categories of biomarkers for the detection of drug-induced nephrotoxicity. They are conventional (traditional (noninvasive)) and novel markers.
- The conventional biomarkers include determination of physiological/metabolic products such as creatinine in urine/plasma and urea in serum, measurement of glomerular filtration rate, evaluation of level of blood urea nitrogen, estimation of concentration of albumin in the urine, urine microscopy, or microscopy of urine sediment.

- Novel biomarkers include neutrophil gelatinase-associated lipocalin, kidney injury molecule 1, liver-type fatty acid-binding protein, calprotectin, brain natriuretic peptide, soluble ST2, galectin-3, urinary microRNA, β2-microglobulin, cystatin C, clusterin which can be used to access the nephrotoxicity.
- Symptoms of nephrotoxicity have a wide range and these can be fatigue, irregular heartbeat, shortness of breath, decreased urine output, nausea, retention of fluid, and swelling in ankles, legs, and feet.
- Diagnostic tests for nephrotoxicity include examination of blood, urine, and serum to check the level of various biological markers. Glomerulonephritis, renal tubule acidosis, and chronic kidney disease are some of the disorders related to nephrotoxicity.
- It is usually suggested that preventive measures should be taken to avoid nephrotoxicity and it includes the dosage of non-nephrotoxic drugs which have high efficacy and least risk factors.

Cross-References

► Recommended Resources for Biomarkers in Disease: Toxicology

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Biomarkers of Tobacco Use: Relevance to Tobacco Harm Reduction

37

Elaine K. Round and Kristin Marano

Contents

Abstract

Cigarette smoking is associated with a large number of adverse health outcomes and continues to be a major contributor to death and disease. Although the best option for cigarette smokers concerned about their health is cessation, tobacco harm reduction is the public health concept that cigarette smokers who are unwilling or unable to quit smoking can switch to alternative non-combustible

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nicotine-containing products in order to reduce or minimize adverse health effects. Long-term epidemiological studies of alternative non-combustible nicotine-containing products are ideal for the evaluation of product use and clinical disease outcomes. However, in many cases such studies are not available. Biomarkers of exposure and potential harm, relevant to both combustible cigarette exposure and smoking-related diseases, are useful in the assessment of potential changes in health effects resulting from switching to alternative non-combustible nicotine-containing products.

Keywords

Tobacco · Nicotine · Harm reduction · Biomarkers of exposure (BoE) · Biomarkers of potential harm (BoPH) · Electronic nicotine delivery systems (ENDS) · Tobacco heating products (THP) · Smokeless tobacco · Snus · Switching · Potentially reduced exposure products (PREP) · Potentially reduced risk products (PRRP) · Oxidative stress · Inflammation

Abbreviations

2,3-d-TXB2	2,3-dinor thromboxane B2
(±)-5-iPF2α-VI	(±)-5-iso-prostaglandin F2α
8-OHdG	8-hydroxy-2′-deoxyguanosine
8-epi-PGF2α	8-iso-prostaglandin F2α
11-DH-TXB2	11-dehydro-thromboxane B2
BoE	Biomarkers of exposure
BoPH	Biomarkers of potential harm
CDC	Centers for Disease Control
ENDS	Electronic nicotine delivery systems
FDA	Food and Drug Administration
ICAM-1	Intercellular adhesion molecule-1
IL-8	Interleukin 8
IL-12	Interleukin 12
IOM	Institute of Medicine
LTE4	Leukotriene E4
NASEM	National Academies of Sciences, Engineering, and Medicine
PHE	Public Health England
PRR	Potentially reduced risk
PRE	Potentially reduced exposure
SAMHSA	Substance Abuse and Mental Health Services Administration
THP	Tobacco heating products
THR	Tobacco harm reduction
US	United States
WBC	White blood cells
WHO	World Health Organization

Introduction

Cigarette smoking continues to be a major cause of preventable morbidity and premature mortality worldwide. Globally, 6.3 million deaths per year are attributed to cigarette smoking (Drope et al. 2018), and in the United States (USA) alone, 16 million individuals are living with smoking-attributable diseases (CDC 2021). Although the prevalence of cigarette smoking has declined in most countries since the 1950s, it has been reported that among those aged 15 years or older, 942 million men and 175 million women are current smokers (Drope et al. 2018, p. 20). In the USA, smoking has decreased significantly over time, i.e., 42% in 1970 and 12.5% in 2019 (Cornelius et al. 2022). However, 34.1 million adults in the USA continue to smoke cigarettes (Cornelius et al. 2022).

Harm Reduction

Generally, "harm reduction refers to interventions aimed at reducing the negative effects of health behaviors without necessarily extinguishing the problematic health behaviors completely" (Hawk et al. 2017, p. 1). That is, harm reduction (also referred to as "risk reduction" and "harm minimization") is a proactive and evidence-based approach to reduce the negative personal and public health impacts of certain behaviors that involve risk (SAMHSA 2022). Examples of harm reduction programs include the enactment of seat belt laws to reduce the harm from driving automobiles, as well as opioid substitution programs for the treatment of illicit drug use (Hawk et al. 2017).

The concept of harm reduction has also been introduced for minimizing the harms related to combustible tobacco use (i.e., tobacco harm reduction, THR) (IOM 2001). The best way to reduce harm associated with cigarettes is to quit smoking. However, for those smokers unwilling or unable to quit, potentially reduced risk and exposure (PRR/PRE) nicotine-containing products are a reasonable alternative for reducing harm related to combustible cigarettes. Although no tobacco product is without risk, the majority of harm associated with tobacco-related disease outcomes are a result of inhalation of smoke from burned tobacco. Public health experts conceptualize the risk differential among nicotine-containing products along a continuum, placing combustible cigarettes on the most harmful end of the spectrum, and non-combustible products (i.e., US/Swedish smokeless tobacco, other nicotine-containing products, and nicotine replacement therapies) on the least harmful end (Fig. 1) (Zeller et al. 2009; Abrams et al. 2018; Hatsukami et al. 2007). THR is a scientific evidence-based approach that can help current smokers stop smoking and thus save lives.

Because tobacco in cigarettes is burned, and the resulting smoke inhaled, smokers are exposed to a substantial number of combustion-related toxicants, as well as other substances that transfer directly from tobacco to smoke. Switching to

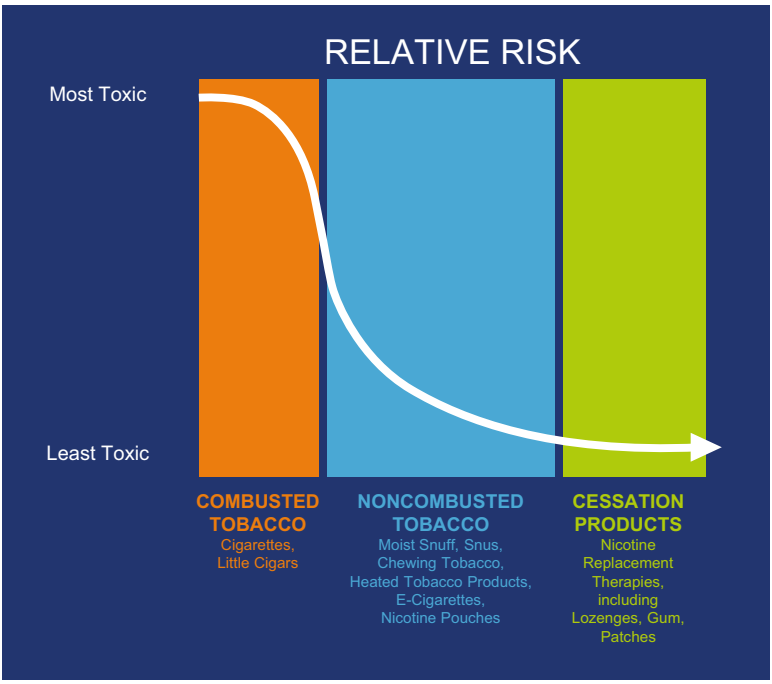

Fig. 1 Continuum of risk associated with nicotine-containing products. (This figure shows the continuum of risk associated with nicotine-containing products. Adapted from Nutt et al. 2014)

non-combustible alternative nicotine-containing products will reduce smoking-attributable exposure and, potentially, risk. PRR/PRE nicotine-containing products include electronic nicotine delivery systems (ENDS), tobacco heating products (THP), smokeless tobacco products (e.g., US and Swedish moist snuff and snus), and oral tobacco products that contain nicotine but do not contain tobacco leaf. These products do not combust tobacco, and thus will not expose consumers to the thousands of toxic chemicals produced from cigarette smoking, including known carcinogens and other respiratory and cardiovascular toxicants. Differences in toxicant exposure create a reasonable expectation of reduced individual disease risk in users of non-combustible tobacco products when compared to combustible cigarette smokers.

Alternative Nicotine-Containing Products

It is generally accepted that use of alternative nicotine-containing products reduce exposure to toxic constituents associated with the combustion of tobacco; there are more than 40 peer-reviewed articles showing such reductions associated with use of

ENDS and THP alone, and at least as many articles showing reductions associated with use of oral smokeless tobacco products. A real-life example comes from Sweden, where there is a tradition of using snus, a low-toxicant oral smokeless tobacco product. In combination with favorable government regulation and a substantial price differential between snus and cigarettes, a rapid rise in the use of snus resulted in a reduction in cigarette consumption. In Sweden, smoking rates have declined from around 30% in the 1980s (Djurdjevic et al. 2019) to approximately 7% in 2019 (Stewart 2020). Notably, these are the lowest rates of smoking among any high-income country (Ramström and Wikmans 2014; Ramström et al. 2016). Further, the lowest level of mortality attributable to tobacco among men in the European Union states has been consistently found in Sweden (Ramström and Wikmans 2014). There are epidemiological data that clearly demonstrate reduced risk of smoking-attributable diseases associated with snus use versus cigarette smoking (Lee 2007; Lee and Hamling 2009). Epidemiological data also demonstrate that oral tobacco product use in the USA (i.e., moist snuff and chewing tobacco), results in reduced risk of cigarette-smoking associated adverse health effects (Henley et al. 2005, 2007). Public health experts agree that certain smokeless tobacco products are associated with less risk. Zeller et al. (2009) note, "Cigarette smoking is undoubtedly a more hazardous nicotine delivery system than various forms of noncombustible tobacco products for those who continue to use tobacco. . . if smokers who cannot or will not quit their dependence on nicotine switched completely to smokeless tobacco products, they would likely experience a reduction in tobacco-caused mortality and morbidity." In October 2019, eight snus products were authorized by the US FDA to be marketed as modified risk tobacco products; these were the first products to be authorized as such (FDA 2019a).

ENDS are another subset of PRR/PRE nicotine-containing products. ENDS have attracted substantial scientific interest since their arrival in 2006. Although long-term epidemiological studies are not yet available, a rapidly growing body of evidence suggests that ENDS products are likely to be far less harmful to individual health than combustible cigarettes. Many public health experts agree (Balfour et al. 2021). It is generally accepted that, except for nicotine, under typical conditions of use, exposure to potentially toxic substances from ENDS is significantly lower compared with combustible cigarettes (NASEM 2018; Balfour et al. 2021). ENDS do not contain or burn tobacco. Instead, a heating component is used to aerosolize a mixture of glycerin, propylene glycol, water, nicotine, and flavoring ingredients. Therefore, users of ENDS are not exposed to combustion-related toxicants that drive the individual disease risks associated with cigarette smoking. For ENDS products, public health experts are moving toward a consensus that ENDS are located at the lower risk end along the nicotine product risk continuum (Fig. 1) (Balfour et al. 2021). A review by the National Academies of Sciences, Engineering and Medicine (NASEM) concluded that "[t]he evidence about harm reduction suggests that across a range of studies and outcomes, [ENDS] pose less risk to an individual than combustible tobacco cigarettes" (NASEM 2018, p. 11). Estimates vary regarding

the extent to which ENDS-related health risks differ from those of combustible cigarettes, but Public Health England (PHE) maintains that "stating that vaping is at least 95% less harmful than smoking remains a good way to communicate the large difference in relative risk unambiguously" (McNeill et al. 2018, p. 20). Further, the Royal College of Physicians similarly concluded that the long-term health risks associated with ENDS "are unlikely to exceed 5% of those associated with smoked tobacco products, and may well be substantially lower than this figure" (RCP 2016, p. 84). Officials at the US FDA have noted that ENDS present a "significant opportunity" for adult smokers to transition away from combustible cigarettes (Zeller 2019, p. S76).

Tobacco heating products (THP) are another set of PRE/PRR nicotine-containing products. These products, also referred to as heated tobacco products, contain nicotine and "do not combust tobacco like cigarettes but heat it to a lower temperature with the aim of avoiding the harmful products of combustion" (McNeill et al. 2018, p. 201). Although THP have been around since the 1980s, they originally were not acceptable to consumers (McNeill et al. 2018). More recently, THP are gaining momentum, particularly in Asia. In Japan, prevalence of THP use has increased from 1.4% in 2015 to 10.9% in 2020 (Igarashi et al. 2021; Odani and Tabuchi 2021). In the US, one THP (i.e., "IQOS") has been authorized by the FDA to be marketed as reduced exposure with specific advertising claims (FDA 2020).

Cigarette Smoking and Disease

The principal disease types associated with cigarette smoking are cardiovascular disease, cancer, and respiratory disease. Chronic cigarette smoking is associated with elevated oxidative stress and inflammation, which are key early drivers of smoking-induced pathophysiology related to these diseases (USDHHS 2010; Chang et al. 2019). Thus, biological markers of oxidative stress and inflammation are likely useful in terms of the understanding of tobacco-related harm, as well as potential changes in harm, related to smoking cessation or switching to PRR/PRE alternative-nicotine containing products.

Epidemiological studies demonstrate that many of the chemical constituents produced by combustion of tobacco and/or transfer from cigarette smoke are associated with adverse health outcomes (Table 1). Accordingly, these exposure markers are useful in the understanding of tobacco-related harm, as well as potential changes in harm, as a result of switching to PRR/PRE nicotine-containing products or quitting altogether. According to Marques et al. (2021, p.1), "[e]xposure to these compounds can be measured using different methods (involving for instance liquid- or gas-chromatographic procedures) in a wide range of biological specimens to estimate the type and degree of tobacco exposure." Table 1 presents a sample of known harmful constituents in tobacco smoke, the associated exposure biomarker that is available to measure the compound, the associated biologic matrix in which it is measured, and the health outcomes associated with these constituents.

Table 1 Sample of biomarkers of tobacco exposure and disease relevance

Constituent	Biomarker	Matrix	Disease Relevance
1,3-butadiene	Monohydroxybutenyl mercapturic acid (MHBMA)	Urine	Carcinogen Respiratory Toxicant Reproductive/ Developmental Toxicant
2-aminonaphthalene	2-aminonaphthalene (2-AN)	Urine	Carcinogen
4-aminobiphenyl	4-aminobiphenyl (4-ABP)	Urine	Carcinogen
o-toluidine	o-toluidine (o-T)	Urine	Carcinogen
Acrolein	3-hydroxypropyl mercapturic acid (HPMA)	Urine	Respiratory Toxicant Cardiovascular Toxicant
Benzene	S-phenyl mercapturic acid (SPMA)	Urine	Carcinogen Cardiovascular Toxicant Reproductive/ Developmental Toxicant Respiratory Toxicant
Ethylene oxide	2-hydroxyethyl mercapturic acid (HEMA)	Urine	Carcinogen Respiratory Toxicant Reproductive/ Developmental Toxicant
Hydrogen cyanide	Thiocyanate (SCN)	Urine	Respiratory Toxicant Cardiovascular Toxicant
4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone [NNK]	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol + glucuronides [Total NNAL]	Urine	Carcinogen
Carbon monoxide	COHb (carboxyhemoglobin)	Blood	Reproductive/ Developmental Toxicant Cardiovascular Toxicant

Source: FDA (2012)
This table presents a sample list of known harmful constituents in tobacco smoke, the associated exposure biomarker that is available to measure the compound, the associated biologic matrix in which the compound is measured, and the health outcomes associated with these constituents

Biomarkers Related to Tobacco Use

Tobacco-related biomarkers have been defined as "measurements of any tobacco constituent, tobacco smoke constituent, or effect of such a compound on a body fluid (including exhaled air) or organ" (IOM 2001). Experts indicate that biomarkers can

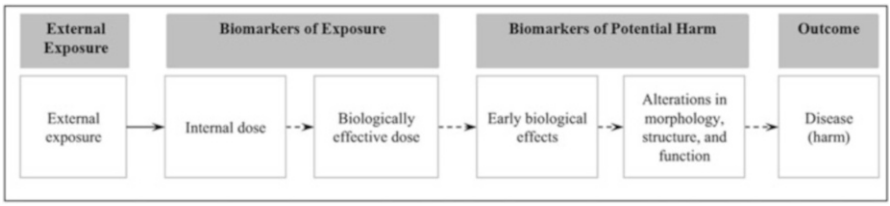

Fig. 2 Relationship of biomarkers with external exposure and disease outcome. (This figure shows the relationships between biomarkers of exposure and biomarkers of potential harm within the continuum of external exposure to clinical disease outcome.)

fall into different categories, for example, biomarkers of exposure; biomarkers of effective dose; biomarkers of potential harm/effect; and biomarkers of biological changes in structure and function (Scherer 2018). However, generally, biomarkers can be separated into two broad groups: biomarkers of exposure (BoE) and biomarkers of potential harm (BoPH) (USDHHS 2010; IOM 2012). Figure 2 characterizes the relationships between BoE and BoPH within the continuum of external exposure to clinical disease outcome. Biomarkers related to tobacco use integrate the variables of product composition and product use behaviors to provide a more accurate assessment of actual human exposure and potential health risk. A number of BoE and BoPH relevant to tobacco use have been identified and can be tested in biological samples such as saliva, blood, urine, and exhaled breath. For tobacco-related BoE, Chang et al. (2017, p. 293) have noted "a broad body of evidence exists for tobacco-related exposure biomarkers, ranging from analytical validation to disease risk." In contrast, viable BoPH related to tobacco use are less definitive, as most of the serious health outcomes caused by smoking arise from cumulative effects that develop over many years, and potential biological markers lack disease and/or tobacco use specificity. Chang et al. (2019) report there are many unanswered questions and little consensus on validated BoPH and tobacco use, aside from the already established BoE with disease relevance. Thus, identifying relevant BoPH is an active area of research. Scherer (2018) notes that a reasonable biomarker associated with tobacco use and changes in tobacco use behavior would possess the following characteristics: (1) association with the disease (Table 1); (2) measurable differences between smokers and non-smokers (Table 2); (3) dose-response relationship; and (4) reversibility upon smoking cessation. Evidence demonstrates that among users of PRR/PRE products, there are improvements in certain biomarkers of oxidative stress and inflammation (Gale et al. 2021a; Haziza et al. 2020a).

Biomarkers of Exposure and Tobacco Use

As noted, an important characteristic of a reliable biomarker is association with disease (Scherer 2018). According to the US Surgeon General, "[d]espite uncertainties about which chemical constituents are responsible for specific adverse health outcomes [associated with cigarette smoking], there is broad scientific agreement

about which chemicals in conventional tobacco-burning cigarettes could be harmful to individuals' health" (USDHHS 2010, Chap. 3). The US Food and Drug Administration Center for Tobacco Products has defined an established list of 93 harmful and potentially harmful constituents in tobacco products along with the disease relevance of each constituent (FDA 2012). A number of compounds have been used as BoE to many of the harmful and potentially harmful cigarette smoke constituents that have been identified in tobacco users including cigarette smokers. Further, these constituents have been associated with various adverse health outcomes (Table 1). Clinically relevant BoE for some of these constituents provide a useful tool for measuring systemic exposure to toxicants present in cigarettes and other tobacco products (NASEM 2018). BoE provide a realistic and direct assessment of toxicant dose for an individual, and are considered accurate and reliable metrics of the levels of exposure that consumers actually experience when using tobacco products (Chang et al. 2017). BoE integrate the specific product being used and product use behaviors to measure an individual's constituent or toxicant exposure over hours, days, or weeks, depending on the specific compound's clearance rate in an individual user, an individual's genetic makeup, and the route of exposure (e.g., inhalation, oral). Notably, individual-specific metabolism, genetics, and route of exposure can influence the interpretation of biomarker data (Gregg et al. 2013). Integration of the specific product being used and product use behavior is a direct measure of exposure as opposed to attempts to estimate exposure via measurements of either the composition of the product itself or product yield assessed via machine smoking methods (Table 2).

Table 2 Sample of biomarkers of exposure and evidence of reliability to distinguish smokers from non-smokers

Biomarker	Constituent	Matrix	Reference
Monohydroxybutenyl mercapturic acid (MHBMA)	1,3-butadiene	Urine	Ding et al. (2009)
2-aminonaphthalene (2-AN)	2-aminonaphthalene	Urine	Riedel et al. (2006)
4-aminobiphenyl (4-ABP)	4-aminobiphenyl	Urine	Riedel et al. (2006)
o-toluidine (o-T)	o-toluidine	Urine	Riedel et al. (2006)
3-hydroxypropyl mercapturic acid (HPMA)	acrolein	Urine	Ding et al. (2009)
S-phenyl mercapturic acid (SPMA)	benzene	Urine	Ding et al. (2009)
2-hydroxyethyl mercapturic acid (HEMA)	ethylene oxide	Urine	Ding et al. (2009)
Thiocyanate (SCN)	hydrogen cyanide	Urine	Scherer et al. (2006)
4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol + glucuronides [Total NNAL]	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone [NNK]	Urine	Chang et al. (2017)
COHb (carboxyhemoglobin)	CO (carbon monoxide)	Blood	Chang et al. (2017)

This table presents a sample list of biomarkers of exposure and the associated references demonstrating their ability to reliably distinguish smokers from non-smokers

Changes in Biomarkers of Exposure Accompanying Changes in Tobacco Use

Clearly, changes or differences in BoE among tobacco users are beneficial in understanding adverse health effects. However, not only would informative biomarkers be characterized by association with disease (Table 1), they would similarly demonstrate measurable differences between smokers and non-smokers, a dose-response relationship, and reversibility upon smoking cessation (Scherer 2018). There is unambiguous evidence that concentrations of some of these tobacco-related BoE decrease following cessation, and demonstrate a dose-response relationship, thus further establishing their utility (Hatsukami et al. 2006). Notably, many of these markers are lower in users of lower-toxicant nicotine-containing products (ENDS, THP, and smokeless tobacco) compared with smokers. Given this, switching to these products is consistent with the concept of THR.

Low levels of harmful or potentially harmful constituents are observed in users of ENDS, THP, and smokeless tobacco, in particular because these products do not combust tobacco. Regarding ENDS, the NASEM has stated, "[t]here is conclusive evidence that completely substituting [ENDS] for combustible tobacco cigarettes reduces users' exposure to numerous toxicants and carcinogens present in combustible tobacco cigarettes" (NASEM 2018, p. 11). Although long-term epidemiological studies are not yet available for ENDS, clinical biomarker studies comparing ENDS use with combustible cigarette smoking consistently show that BoE levels are markedly lower when consumers use ENDS or when cigarette smokers switch to ENDS (Hecht et al. 2015; Goniewicz et al. 2017; Shahab et al. 2017; Round et al. 2018). In addition, studies have demonstrated that smokers who completely substitute combustible cigarettes with ENDS experience reductions in toxicant exposure similar to smokers who quit smoking over the same period of time as measured by BoE. Further, evidence indicates a comparable magnitude of reductions as observed when smokers switch to nicotine gum or abstinence (O'Connell et al. 2016; Round et al. 2018; McEwan et al. 2021).

Similarly, long-term epidemiological studies are not yet available for THP. However, reductions in harmful constituents are observed in users of THP compared with cigarette smokers. A report commissioned by PHE concluded "[c]ompared with cigarettes, heated tobacco products are likely to expose users and bystanders to lower levels of particulate matter and harmful and potentially harmful compounds (HPHC). The extent of the reduction found varies between studies" (McNeill et al. 2018). Human studies, including randomized controlled trials, demonstrate lower levels of BoE among smokers who switched to THP compared with those who continued smoking (Haziza et al. 2016a, b; Auer et al. 2017; Lüdicke et al. 2017, 2018; Haziza et al. 2020b; Gale et al. 2021a, b) and among switchers (Ogden et al. 2015a).

Finally, data demonstrate that BoE associated with cigarette smoking are lower in users of smokeless tobacco compared with smokers (Naufal et al. 2011; Campbell et al. 2015; Rostron et al. 2015; Prasad et al. 2016; Cheng et al. 2020), and switching from combustible cigarette smoking to use of oral smokeless tobacco (Krautter et al. 2015; Ogden et al. 2015a) results in decreased exposure to combustible tobacco toxicants. For smokeless tobacco in particular, the differences in chemistry,

combined with differences in the route of exposure, allow users to reduce or eliminate exposures to many combustion products and eliminate direct exposure of lung tissues to the harmful effects of those compounds.

Thus, consumers who switch from combustible cigarette smoking to alternative PRR/PRE nicotine-containing products reduce their exposure to harmful constituents. Such reductions in exposure are likely to reduce individual health risks given the known adverse health effects associated with such constituents. This switching is consistent with the concept of THR.

Biomarkers of Potential Harm

The optimal method of measuring reduced harm is via long-term epidemiological studies evaluating tobacco use behavior and clinical disease outcomes. However, given the relatively short duration that some PRE/PRR (e.g., ENDS, THP) products have been available in the market, as well as the long duration necessary for chronic disease progression and development, it is important to identify interim measures (i.e., BoPH) to evaluate the effect of changes in tobacco use behavior on chronic smoking-related diseases.

Generally, a BoPH reflects biological changes that may result from toxicant exposure and are selected to indicate potential changes along a disease pathway prior to the manifestation of disease (USDHHS 2010; IOM 2012). According to the US Surgeon General, "[m]arkers of health effects reflect preclinical changes short of those reached when clinical disease occurs. . .A biomarker of a biologic event with the potential to lead to harm [BoPH] is defined as a measurement of an effect attributable to exposure, including early biologic effects; alterations in morphology, structure, or function; and clinical symptoms consistent with harm. In the more general formulation, such biomarkers constitute markers of health effects" (USDHHS 2010, Chap. 3).

Inflammation and oxidative stress are associated with major smoking-attributable diseases, including heart disease, cancer, and respiratory disease (USDHHS 2010). In addition, clinical studies that evaluate BoPH show that combustible cigarette smokers experience measurable and significant changes in molecular, cellular, and physiological systems that reflect damage or potential damage from cigarette smoke resulting in oxidative stress and inflammation (USDHHS 2010). In smokers, these changes can be related to increases in risks for cancers, non-neoplastic pulmonary disease, cardiovascular disease, and a variety of other adverse health effects (USDHHS 2010). While few if any of the changes are specific to cigarette smoking, smoking's effects on oxidative stress and inflammation are all evident in various BoPH. It is reasonable to consider BoPH that reflect early changes in certain biochemical pathways, such as those involved in inflammation or oxidative stress (i.e., known pathophysiology in smoking-related diseases) and are demonstrated to be altered in cigarette smokers. As noted, there is currently no single accepted BoPH that predicts the risk of disease in individuals who use tobacco products. However, evidence demonstrates that among users of PRR/PRE products, there are improvements in certain biomarkers, including those indicative of oxidative stress and inflammation.

Changes in Biomarkers of Potential Harm Accompanying Changes in Tobacco Use

Specific markers associated with cigarette smoking, smoking-related adverse health outcomes, and inflammation and/or oxidative stress include:

- White blood cells (WBC) (Frost-Pineda et al. 2011; Marano et al. 2015; Ogden et al. 2015b; Scherer 2018)
- Intercellular adhesion molecule-1 (ICAM-1) (Ogden et al. 2015b; Scherer 2018; Xue et al. 2019; Akiyama and Sherwood 2021)
- F2-isoprostanes (e.g., 8-iso-prostaglandin F2α [8-epi-PGF2α]; (±)-5-iso-prostaglandin F2α [(±)-5-iPF2α-VI]) (Ogden et al. 2015b; Scherer 2018)
- Arachidonic acid metabolites (i.e., leukotriene E4 [LTE4] and 2,3-dinor-thromboxane B2 [2,3-dinor-TXB2], 11-dehydro-thromboxane B2 [11-DH-TXB2]) (Lowe et al. 2009; Scherer 2018; Makena et al. 2019; Akiyama and Sherwood 2021)
- Interleukins (e.g., IL-8, IL-12) (Nordskog et al. 2015; Scherer 2018)
- DNA adducts (e.g., 8-Hydroxy-2′-deoxyguanosine [8-OHdG]) (Shepperd et al. 2015; Scherer 2018; Akiyama and Sherwood 2021)

These BoPH may be useful as interim health-related measures among tobacco users. Human studies demonstrate that these BoPH have been shown to be changed in smokers compared with non-smokers (Frost-Pineda et al. 2011; Scherer 2018) and as a result of alteration in cigarette smoking behavior, including cessation (Scherer 2018; Xue et al. 2019; Halvorsen et al.), variations in cigarette smoking frequency (i.e., exhibit a dose-response relationship) (Hatsukami et al. 2006; Scherer 2018), and switching to lower toxicant alternative nicotine-containing products (Hatsukami et al. 2006; Krautter et al. 2015; Nordskog et al. 2015; Shepperd et al. 2015; Ogden et al. 2015b; Lüdicke et al. 2018; Round et al. 2018; Makena et al. 2019; Haziza et al. 2020a; Akiyama and Sherwood 2021; Gale et al. 2021a). "Longer-term" BoPH are marked by changes measured after 2 weeks to 6-plus months of product switch. These include (±)-5-iPF2α-VI, 8-OHdG, WBC, and ICAM-1 (Ogden et al. 2015b; Scherer 2018; Haziza et al. 2020a; Akiyama and Sherwood 2021; Gale et al. 2021a). "Shorter-term" BoPH, are marked by changes measured after 5 or 7 days of product switch. These include IL-8, IL-12, and LTE4 (Nordskog et al. 2015; Makena et al. 2019). 2,3-dinor-TXB2, 11-DH-TXB2 and 8-epi-PGF2α have shown changes in both short-term and long-term studies (Shepperd et al. 2015; Scherer 2018; Makena et al. 2019; Haziza et al. 2020a; Gale et al. 2021a).

Cross-sectional analyses indicate that concentrations of these specific BoPH are higher in smokers compared with non-smokers (Koundouros et al. 1996; Lowe et al. 2009; Frost-Pineda et al. 2011; Scherer 2018) and higher in smokers compared with users of non-combustible lower-toxicant nicotine-containing products, including ENDS (Benowitz et al. 2020; Oliveri et al. 2020; Akiyama and Sherwood 2021; Christensen et al. 2021; Stokes et al. 2021), THP (Lüdicke et al. 2018; Akiyama and Sherwood 2021; Sakaguchi et al. 2021), and smokeless tobacco (Marano et al. 2015; Prasad et al. 2016). Similarly, evidence shows these markers may not be different in non-users as compared to alternative nicotine-containing product users, including

ENDS (Benowitz et al. 2020; Christensen et al. 2021), THP (Lüdicke et al. 2018), and smokeless tobacco (Marano et al. 2015; Prasad et al. 2016).

Conclusions

Cigarette smoking remains a major cause of morbidity and mortality globally. In the absence of complete cessation, for smokers unwilling or unable to quit, public policy that advocates THR can have meaningful effect toward reducing or minimizing smoking-attributable death and disease.

BoE and BoPH have been demonstrated to be useful in the assessment of health risks associated with various tobacco products, as well as changes in tobacco use behavior. A reasonable biomarker associated with tobacco use would show: (1) association with the disease; (2) measurable differences between smokers and non-smokers; (3) dose-response relationship; and (4) reversibility upon smoking cessation. Numerous tobacco-related BoE are associated with smoking-attributable adverse health outcomes including cancer, cardiovascular disease, and respiratory disease. Given the many toxicants present in combustible cigarettes, reducing exposure is associated with reducing harm. Consumers who switch from combustible cigarette smoking to alternative PRR/PRE nicotine-containing products reduce their exposure to harmful constituents, and such reductions in exposure are likely to reduce individual health risk. Further, although specific BoPH are not yet available, there are certain markers that show improvements among cigarette smokers who switch to alternative nicotine-containing products. This investigation of relevant BoPH is an active area of research, as long-term epidemiological studies may not be available for many years and interim health markers are important in these assessments.

Reducing the harms associated with combustible cigarette smoking continues to be a public health goal across the world (WHO 2003; FDA 2019b). There is no safe tobacco product and quitting smoking is the best way to reduce the risk of developing smoking-related diseases. However, THR is becoming an accepted strategy among many public health experts for those smokers unwilling or unable to quit (NICE 2013; FDA 2017). In the USA, the FDA has the ability to authorize the marketing and labeling of certain tobacco products as reduced exposure or reduced risk. The marketing of eight smokeless tobacco products and four THP products have been authorized as such, and manufacturers can advertise these products with specific reduced exposure or reduced risk claims. Thus, tobacco manufacturers also have a role in minimizing harms caused by cigarette smoking as they continue to develop innovative PPR/PPE products that facilitate an acceleration in the decline of smoking prevalence. It is clear that THR is a public health policy that will help to save lives.

Applications to Prognosis

Epidemiological evidence is available that demonstrates the use of US and Swedish smokeless tobacco products is associated with less harm than combustible cigarette smoking. That is, lung cancer, oral cancer, respiratory disease, and cardiovascular

disease risks are lower in smokeless tobacco users compared with cigarette smokers. Knowing this, differences in biomarkers among smokeless tobacco users compared with cigarette smokers is a way to begin to identify biomarkers relevant to disease that can be used in scenarios where long-term epidemiological data are not available. Such biomarkers could also be applied to users of newer non-combusted tobacco products to assess risk and the potential for harm reduction.

Applications to Other Diseases or Conditions

Individual BoE and BoPH, associated with smoking-attributable diseases, have been associated with tobacco use itself. These markers have been shown to change in response to changes in tobacco use, consistent with the concept of tobacco harm reduction. Given this, the use of these types of biomarkers can likely be applied to evaluate the benefits of broader THR policies.

Mini-Dictionary of Terms

- ***Biomarkers of potential harm (BoPH)***. *Biological markers of health effects that represent preclinical and/or early biological changes.*
- ***Electronic Nicotine Delivery System (ENDS)***. *Inhalation devices that aerosolize a mixture of glycerin, propylene glycol, water, nicotine, and flavoring ingredients. ENDS do not contain or burn tobacco.*
- ***Tobacco Heating Product (THP)***. *Nicotine-containing inhalation devices that heat, rather than combust, tobacco thus avoiding inhalation of the toxic products of combustion.*
- ***Tobacco Harm Reduction***. *Changes in tobacco use behavior, including switching to lower-toxicant alternative nicotine-containing products, which will minimize smoking-attributable harm without eliminating nicotine use.*
- ***Longer-term BoPH***. *BoPH for which changes are observed 2-weeks to 6+ months after product switch.*
- ***Potentially reduced risk and exposure (PRR/PRE) products***. *Nicotine-containing products that are a reasonable alternative for reducing harm related to combustible cigarettes.*
- ***Shorter-term BoPH***. *BoPH for which changes are observed after 5 to 7 days of product switch.*

Key Facts of Cigarette Smoking and Biomarkers of Exposure and Potential Harm

- Globally, 6.3 million deaths per year have been attributed to cigarette smoking-related diseases, including heart disease, respiratory disease, and cancer.
- Harm reduction is the concept of reducing adverse health effects associated with certain behaviors where the associated behavior is not completely eliminated.

- Tobacco harm reduction is the concept that changes in tobacco use behavior, including switching from combustible cigarettes to lower-toxicant alternate nicotine-containing products, will minimize smoking-attributable harm without eliminating nicotine use.
- In the absence of long-term epidemiological studies, biomarkers related to tobacco use can be used to predict adverse health outcomes, including changes in adverse health outcomes.
- Experts have noted that relevant biomarkers related to tobacco use would have the following characteristics: (1) association with the disease; (2) measurable differences between smokers and non-smokers; (3) dose-response relationship; (4) and reversibility upon smoking cessation.

Summary Points

- Cigarette smoking is associated with multiple adverse health outcomes and continues to be a major contributor to death and disease.
- The best option for cigarette smokers concerned about their health is to quit smoking; however, tobacco harm reduction is the public health concept that cigarette smokers who are unwilling or unable to quit can switch to alternative, lower toxicant nicotine-containing products in order to reduce or minimize adverse health effects.
- Biomarkers of exposure and biomarkers of potential harm relevant to both combustible cigarette smoking exposure and smoking-related disease are useful in the assessment of potential changes in health effects resulting from switching to alternative nicotine-containing products.
- Consumers who switch from combustible cigarette smoking to non-combustible alternative nicotine-containing products reduce their exposure to harmful constituents. Such reductions in exposure are likely to reduce individual health risks given the known adverse health effects associated with such constituents.

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Bleaching Agents as Toxic Compounds and Biomarkers of Damage

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Abstract

Bleaching materials such as hydrogen peroxide (HP) and carbamide peroxide (CP) have been utilized within aesthetic dentistry by dental practitioners for more than a century to achieve teeth-lightening treatment. The safety of these agents, which can be applied with or without light, for different durations and forms of administration, has been a source of debate and concern. Reactive oxygen species released in the reaction of peroxide-derived materials used during bleaching treatment cause side effects such as genotoxicity, cytotoxicity, DNA damage, gingival irritation, and the most common tooth sensitivity besides the whitening in enamel surfaces. Biological and pathogenic processes can be followed by using various biomarkers to identify the detrimental effects of these released by-products. Biological properties and toxicology of HP and similar bleaching agents, including safety studies of teeth whitening and clinical evaluations of its use and also the safety aspects of teeth bleaching treatment, are reviewed within this chapter.

Keywords

Tooth bleaching · At-home · In-office · Carbamide peroxide · Hydrogen peroxide · Biomarker · Reactive oxygen species · Papain · Bromelain · Ficin · Nanoparticles · TiO_2 · Chitosan

List of Abbreviations

8-OH-dG	8-hydroxy-2′-deoxyguanosine
8-OHGua	C8-hydroxyguanine
CAT	Catalase
ClO_2	Chlorine dioxide
CP	Carbamide peroxide
EPR	Electron paramagnetic resonance
GPx	Glutathione peroxidase
GR	Glutathione reductase
GST	Glutathione S-transferases
HA	Hydroxyapatite
HP	Hydrogen peroxide
IFNγ	Interferon-γ
IL-1	Interleukins-1
IL-1ß	Interleukin-1β
IL-6	Interleukins-6
LMWA	Low-molecular-weight antioxidants
MBS	Sodium metabisulfite
MDA	Malondialdehyde
NH_2	Amino
NO	Nitric oxide
N_TiO_2	Titanium dioxide doped with nitrogen

ROS	Reactive oxygen species
O_2	Molecular oxygen
$O_2^{-\bullet}$	Superoxide
O_3	Ozone
$OH^{\bullet}$	Hydroxyl radicals
OH^-	Hydroxyl ions
$ONOO^-$	Peroxynitrite
RANKL	Receptor activator of nuclear factor kappa-B ligand
SOD	Superoxide dismutase
TiO_2	Titanium dioxide
TNF-α	Tumor necrosis factor-α
TNF-β	Tumor necrosis factor-β
UV	Ultraviolet

Introduction

Due to the increase in the aesthetic expectations of the patients, aesthetic dentistry has developed considerably in recent years. To ensure the smile aesthetics on a person's face, conventional dental treatments for the appearance such as veneer or crowns started to be replaced with minimally invasive treatments by dentists and clinicians. Tooth bleaching, which is one of these minimally invasive treatments, is widely used for discolorations to maintain the aesthetics (Dahl and Pallesen 2003). Discoloration of the tooth is classified as intrinsic, extrinsic, and a combination of both which depend on its etiology, localization, how deep into the tooth structure, and severity (Hattab et al. 1999). Intrinsic discoloration is the color change that occurs due to the integration of chromogenic materials (caused by such as prolonged tetracycline therapy, high-dose fluoride exposure, and trauma) inside the dentin and enamel structure among tooth formation and/or following an eruption. Various foods and beverages and long-term use of cigarettes can be the causes of extrinsic discoloration (Hattab et al. 1999; Watts and Addy 2001). Bleaching treatment may be performed externally or internally (intracoronary area) known as, respectively, vital tooth bleaching and non-vital tooth bleaching.

Vital tooth bleaching treatment has several techniques and approaches such as bleaching agent's concentration, type of agents, duration of application, application mode, and activation with or without light (Cintra et al. 2013; Dahl and Pallesen 2003). There are three different procedures in use: at-home bleaching, in-office bleaching, and combination of both techniques (Auschill et al. 2005; Cintra et al. 2013; Dahl and Pallesen 2003). For at-home bleaching, it is recommended to utilize low-concentration agents within a long period. However, for in-office bleaching, high-concentration agents are used along the shorter application time (Auschill et al. 2005).

Biomarker is reported as a characteristic which is objectively measured and evaluated as a marker of regular biological procedures, pathogenic procedures, or pharmacological responses into therapeutic intervention by the National Institutes of

Health's Biomarkers Definitions Working Group (Downing 2001). Biomarkers have various structures such as DNA methylation, gene mutations, histone code, gene transcript, miRNA, protein product or posttranslational modifications, and also metabolites (Lewin and Weiner 2004). Biomarkers can be applied to develop a diagnosis, for authorized earlier and more precise recognition or prognosis, and also to remark disease progress and/or therapeutic response (Wulfkuhle et al. 2003).

Chemical Bleaching Agents

Hydrogen Peroxide

Hydrogen peroxide (HP) as an oxidizing agent is widely used in lots of different areas: bleaching of textiles, hair, food, fur, and teeth, refinement of water and sewage, or as a neutralizing product within wine distillation. HP is a colorless and pungent liquid, being highly soluble in water. Furthermore, depending on concentration, it can be released in water and forms a pH acidic solution (Walsh 2000). It can be found in humans, plant tissues, bacteria, foods, and beverages (IARC 1985). HP is an unstable structural complex that breaks down into reactive oxygen radicals and water (Walsh 2000).

Carbamide Peroxide

Carbamide peroxide (CP) is a stable structural compound that decomposes into its active components as it reacts with water (Price et al. 2000). Unlike HP, due to its structural stability, slow degradation results in a prolongation of the bleaching reaction process (Walsh 2000). The chemical structure of CP include HP and urea; of note, urea is degraded to carbon dioxide and ammonia (Dahl and Pallesen 2003; Hermans et al. 2007). Therefore, the basic active agent for the tooth bleaching in CP complex is also HP. Besides, urea particles may facilitate diffusion of bleaching agents through the enamel by reducing the organic matrix inside the enamel (Hermans et al. 2007).

Chemical Mechanism of Tooth Bleaching

Bleaching agent products in the market may include HP as an active whitening particle, with glycerin as the carrier, Carbopol as the thickening agent, and various flavoring agents (ADA 2005). The bleaching procedure is performed by breaking down chromogens that caused the discolored tooth structure. Chromogens generally resided either as large double-bonded organic complexes or as metallic-including complexes that are more difficult to whiten using HP.

Reactive oxygen species (ROS) which may also become intercellular and intracellular messengers are free radicals and reactive molecules produced by molecular

oxygen (O_2). These free radicals and reactive molecules generally composed of hydroxyl radicals ($OH^{\bullet}$), HP, nitric oxide (NO), hydroxyl ions (OH^{-}), peroxides, peroxynitrite ($ONOO^{-}$), singlet oxygen, and superoxide ($O_2^{-\bullet}$) (Atashi et al. 2015).

During the tooth bleaching, ROS derived from HPs react with chromogens to break the double bond of organic structures, reacting with the simple molecules, destabilizing the chromogenic complex, and eventually reducing the discoloration over time (Carey 2014).

At the low levels, ROS are enhanced in the growth, repair of tissue damage, cell proliferation, and angiogenesis which are beneficial for cells (Yasuda et al. 1998). Conversely, high levels of ROS can produce destructive effects leading to cell death, apoptosis, genotoxicity, and cytotoxicity. According to a study (Lee et al. 2006), there was an effect of HP on odontoblast, and using 0.3 mmol/L of HP, ROS accumulation, which facilitates cell differentiation, was detected in cells.

Toxicity of Tooth Bleaching

According to a report of the Agency for Toxic Substances and Disease Registry in the USA, exposure to high concentrations (>10%) of HP has the potential to irritate body parts such as the skin, eyes, and mucous membranes (ATSDR 2014). The maximum concentration of HP that can be applied to the mucosa without causing any irritation is known as 5%. In addition, HP at concentrations above 50% is considered a corrosive substance. Based on this information, the toxicity of bleaching gels is based on their concentration, components of the agent, and the application duration (Carey 2014). After application of the low-molecular-weight agent, its penetration into the pulp chamber and the periodontal ligament causes an inflammatory reaction which may initiate cervical root resorption and detriment into the pulp chamber, fibroblasts, or DNA structure (Bahuguna 2013). Although it is known that ROSs presented genotoxicity and cytotoxicity, it has been reported that radicals cannot pass through cell membranes and cause damage unless HP is applied at 30% or higher concentrations (Tredwin et al. 2006).

Natural Alternative Bleaching Agents

In dentistry, as a bleaching agent generally, CP and HP are used. In some cases, sensitivity was reported by many patients after the bleaching treatment (Vaez et al. 2018). The sensitivity after bleaching is thought to be caused by HP molecules. As a consequence of HP diffusion into the enamel and dentin, by-products released after the reaction may spread into the pulp chamber (Shackelford et al. 2000) and produce severe detriment to the odontoblasts in particular (Li and Greenwall 2013). Once stimulating the release of pro-inflammatory cytokines, this can affect regenerative potency of pulp (Attin et al. 2005; Soares et al. 2019).

Besides the sensitivity, even after 6 months, tooth bleaching has been related to bone inflammation and resorption procedures, depending on the enhancement of

receptor activator of nuclear factor kappa-B ligand (RANKL) and interleukin-1β (IL-1ß) levels (Bersezio et al. 2019).

In literature, to reduce these side effects on pulp and enamel tissues, there are many new natural experimental or newly developed bleaching (Gimeno et al. 2008; Münchow et al. 2016), which can produce oxidative reactions and remove the discolorations without detrimental consequence (Gopinath et al. 2013; Münchow et al. 2016).

Organic products from raw fruits such as lemon, sweet orange, and white grapefruit have shown proficiency in stabilized or developed tooth color (Violeta et al. 2010). To maintain better or comparable aesthetic outcomes, and reduce the toxic side effects, using these products can be effective.

Some plant-derived enzymes of natural compounds such as polyphenol peroxidase, catalase (CAT), superoxide dismutase (SOD), papain, and bromelain (Gopinath et al. 2013; Münchow et al. 2016) and also bovine milk lactoperoxidase, horseradish peroxidase, glucose oxidase, d-(+)-glucose monohydrate (Gimeno et al. 2008), and chlorine dioxide (ClO_2) (Ablal et al. 2013) can replace the conventional bleaching agents. Nevertheless, the effects of these enzymes were not investigated and well known adequately.

Papain, Bromelain, and Ficin

Bromelain and papain that demolish macromolecules into smaller particles are cysteine proteases. Unlike the macromolecules, smaller particles can be released through interprismatic area, consent the light reflect from the buccal tooth surface, and increased the whitening effect (Sato et al. 2013). According to a research (Soares et al. 2019), enzymatic reaction with peroxidase of these particles developed the biocompatibility and whitening effect of the peroxide-based agents.

Ribeiro et al. (Ribeiro et al. 2020) evaluated the effects of papain-based, ficin-based, and bromelain-based bleaching agents with whitening, microhardness, surface roughness, and cell viability on mouse fibroblast, according to CP. In the terms of bleaching, bromelain-based, ficin-based, and CP bleaching gels demonstrated a similar color change. These new natural agents did not affect the cell viability. Consequently, bromelain-, papain-, or ficin-based bleaching gels can be used to develop nonperoxide gels.

According to Münchow et al. (2016), papain and bromelain were evaluated as regards color change compared to CP. Even though CP showed a better result in whitening, it is substantial to mention that the concentration of bromelain and papain was 20 times lower than peroxide gel.

Chlorine Dioxide

Another bleaching agent that is an alternative to HP was ClO_2.

It was found that ClO_2 showed faster results in the whitening process compared to HP (Ablal et al. 2013). In this study, the bleaching obtained with ClO_2 after 2 minutes

of application using a lightening device showed better color lightening compared to the treatment without light. However, after 30 min of application, there was no improvement in the color change. In addition to these, it is not recommended to use products with lower pH levels with prolonged exposure to the tooth surface. Within our knowledge, there was no other study evaluating the effect of ClO_2.

Sodium Chloride and Sodium Bicarbonate

In a study (Miglani et al. 2012), to improve the efficacy of sodium chloride or sodium bicarbonate agents, it was dissolved with vinegar. The effectiveness of sodium chloride with vinegar including 4% acetic acid is not clear. According to another study (Zheng et al. 2014), comparing the HP, vinegar decreased the microhardness values.

Natural Extract Combined with Hydrogen Peroxide

In literature reviews, natural extracts combined with HP showed more effective lightening compared with HP only. Gimeno et al. (2008) evaluated that association of lactoperoxidase with CP and HP bleaching agent is effective to lighten discoloration due to tetracycline. After an 8-h exposure of lactoperoxidase, whitening efficacy was better than CP itself (Gimeno et al. 2008).

The working principle of lactoperoxidase is to catalyze the decolorating and decomposition reaction of tetracycline with HP. By-products of CP after degradation do not affect the oxidative reaction; however, in these collaborations occurring, lipoperoxidase can let carbamide peroxide particles react with the tetracycline (Gimeno et al. 2008). Consequently, the increased level of oxidative reaction by reacting by-products leads to further lightness (Gimeno et al. 2008).

Another research demonstrated that addition of sweet potato extract into HP has better results compared with the control group: without sweet potato extract, only HP (Gopinath et al. 2013). Enzymatic activity of sweet potato extract containing CAT and peroxidase increases the rate of free radical release by promoting a decrease in activation energy (Gopinath et al. 2013). Thus, the detrimental effects of free radicals on enamel, which may be eliminated for entity of enzymatic and non-enzymatic antioxidants within sweet potato extract, are limited (Gopinath et al. 2013).

There is a study conducted that peroxidase can stimulate the oxidative intermediate product along with the degradation step using one electron, known as peroxidase cycle (Soares et al. 2019). The peroxidase cycle may induce oxidation react rate in HP, increasing the lightness efficiency on enamel surface and decreasing the harmful side effects on the pulp chamber (Soares et al. 2019).

In a study by Marquillas et al. (2020), sodium metabisulfite (MBS) as reducing agent and tannic acid solution as model staining solution were used and compared among the control group, CP. MBS, a well-known food additive, can potentially saturate the double bonds of stained molecules, break down through their

aromaticity, and eventually lighten the discoloration. Tannic acid is a polyphenol that dissolves in water and produces a brown colored solution when dissolved. Polyphenols require oxidation to maintain their ecological activities (Appel 1993) and are also susceptible to oxidation in solution, thanks to their antioxidant activities, radical scavenging, and metal-chelating activities (Badhani et al. 2015). Consequently, MBS showed better and/or equal results in lightning effectiveness.

Nanoparticle-Added Bleaching Agents

Polymeric Nanoparticle

Favoreto et al. (2021) evaluated the bleaching effectiveness and sensitivity through penetration inside the pulp chamber using 15% CP containing polymeric nanoparticles as gel carriers with different durations of application. After 30 and 45 min of application, there was a significant difference between polymeric nanoparticle-added CP experimental gels and conventional CP, which found a higher amount of HP within the pulp tissue after applying conventional gel (Favoreto et al. 2021).

The bleaching process can result in tooth sensitivity after the application of gel, the most common side effect of it (Rezende et al. 2016). Even though the mechanism of sensitivity is not well known, it has been estimated that it affects the pulp tissue through dentin due to increased enamel permeability after bleaching (Kwon and Wertz 2015). Bleaching causes the expression of inflammatory mediators of P-substance (Caviedes-Bucheli et al. 2008) and prostaglandins that trigger pain via nociceptive stimuli, which are responsible for the inflammatory response of tooth sensitivity (Soares et al. 2014). It also causes oxidative stress and even cellular detriment (Vaz et al. 2016). To prevent these deleterious effects, bleaching agents with HP have to be balanced and stabilized.

Therefore, nanomaterials with different properties and advantages have been presented by researchers (Genari et al. 2017; Padovani et al. 2015). Nanotechnology can be useful to reduce the concentration and release rate of HP and also can increase the materials' bioavailability and stability (Padovani et al. 2015). Polymeric nanoparticles as a carrier for the agents were formulated with the distribution of polymeric nanoparticles inside CP (Ramos and Lima 2016).

Besides the better stability and lightening effect of the nanoparticles, as indicated within the research of Lima et al. (Lima et al. 2019), CP nanoparticles provide controlled release of HP, especially in certain regions among the pulp cavity. Eventually, bleaching-induced sensitivity may decrease. CP polymeric nanoparticle experimental gels form a larger molecule, resulting in higher surface area/volume ratio and low polydispersity index, improving their biodistribution, solubility, and interaction of complexes at target sites (Besinis et al. 2015). Polymeric nanoparticles help in faster and easier decomposition of HP, which reduce the HP rate to reach out to pulp tissue and decrease bleaching-induced sensitivity (Favoreto et al. 2021). Lima et al. (2019) demonstrated that there was no inflammatory process within pulp tissue after bleaching with polymeric nanoparticle-added CP.

Nitrogen-Doped Titanium Dioxide

To lower the concentration of HP on behalf of ensuring further lightness and safety, semiconductor agents were used such as photosensitive complexes to absorb supplemental energy through light devices (titanium dioxide doped with nitrogen nanoparticles, N_TiO_2). Consequently, HP reaction can be catalyzed and accelerated (Bortolatto et al. 2013).

Titanium dioxide (TiO_2) is a low-cost photocatalyst that reacts with ultraviolet (UV). The reason TiO_2 is considered safe is that it formed $O_2^{-\bullet}$ instead of $OH^{\bullet}$ which causes related bleaching-induced sensitivity (Saita et al. 2012). However, it should be noted that this formulation has a disadvantage because of the potential biological adverse effect that can be brought about by the dependence on UV radiation to activate the bleaching agent (Soares et al. 2014). To avoid these potential side effects, a new formula was presented where N_TiO_2, which allows the use of wavelengths of visible light, avoids UV radiation. N_TiO_2 nanoparticles added to HP have demonstrated further efficiency along with blue (wavelength between 480 nm and 520 nm) and violet LED lights (Bortolatto et al. 2014).

Although the use of infrared lasers is allowed to reduce the bleaching-induced immediate sensitivity, these results might not be relevant due to the use of low-potency (100 mW ± 20%) lasers (Martin et al. 2015; Vildosola et al. 2017). There is no evidence about positive effect of applied different power and wavelength lasers on the catalysis of the bleaching reaction.

Vildosola et al. (2017) demonstrated that low concentrations of 6% HP presented admissible levels of lightness efficacy and practically no sensitivity. Mechanisms of TiO_2-added bleaching agents remain vague (Bortolatto et al. 2016). However, Matis et al. (2009) found that to maintain the effective lightness with 6% HP, the agent must be applied for 20 hours.

In a recent study by Bersezio et al. (2021), it has been determined that the application of LED/laser lamp infrared together with 6% HP with TiO_2 provides increased efficiency for the study groups by acting as a catalyst.

Coto et al. (2017) demonstrated that TiO_2/Ag nanoparticles can be further and better activated than only TiO_2 nanoparticles, with UV and visible light containing blue light. Based on these results, Kurzmann et al. (2019) evaluated the viability of L-929 cells, 3 T3 cells, and gingival fibroblast after bleaching application using 6% TiO_2/Ag nanoparticles. Consequently, it was found that experimental bleaching gels including TiO_2 and/or TiO_2/Ag demonstrated less cytotoxicity than gel including HP. In addition, as the empty gel and the gel including TiO_2 had a similar reaction in terms of toxicity, the gel including TiO_2/Ag was found to be more cytotoxic (Kurzmann et al. 2019).

Nano-bioactive Material

During bleaching treatment, diffusion of HP may be increased due to microscopic defects such as porosity (Akal et al. 2001) on the enamel surface, resulting in a reduction in enamel microhardness (da Costa Soares et al. 2013). In addition, some studies have demonstrated that cracks and/or fissures (Benetti et al. 2004; Gökay et al. 2000) significantly increase tooth sensitivity in patients as they facilitate

diffusion of HP inside the pulp chamber (Özcan et al. 2014). Therefore, to reduce bleaching-induced sensitivity and decrease the diffusion of HP, agents can be used to repair microscopic defects. There are so many bioactive materials in clinical use to reduce sensitivity such as ACP (amorphous calcium phosphate), CPP-ACP (ACP with the casein phosphopeptide) (Maghaireh et al. 2014), HA (hydroxyapatite) (Vano et al. 2015), and other bioactive glasses.

In many studies (Alexandrino et al. 2017; Maghaireh et al. 2014; Pintado-Palomino et al. 2015), these compounds have been used by incorporating them into pastes and/or mousses before application or after bleaching, but this increases the operator's working time and complicates procedure.

Bioglass, one of the bioactive materials, is composed of amorphous ceramic material (F 1989), and the mechanism of action, nucleation, and precipitation of calcium and phosphate ions increase the deposition of HA upon tooth surface, providing restoration (Forsback et al. 2004), remineralization (Li 2006), and enamel surface repair (Bakry et al. 2011). Bioglass can have high bioactivity when used in nanoparticle form (Ajita et al. 2015).

Burey et al. (2021) demonstrated that bleaching agents which include bioactive materials for in-office treatment did not affect the lightness effectiveness and tooth sensitivity among study groups. However, for in-office bleaching, risk of sensitivity was low in both groups. Apart from that, only two studies (Borges et al. 2012; Vano et al. 2015) have performed a blend of bioactive materials within a commercial agent toward in-office bleaching.

Chitosan

Chitosan is a derivative from chitin with a deacetylation procedure and contains high amounts of amino (NH_2) and OH^- functional groups within its structure. It has capability to adsorb dyes (Chatterjee et al. 2011), metal ions (Wang et al. 2013), and organic acids (Cruz-Romero et al. 2013). It has many properties to maintain its use in biomedical applications such as being non-toxic, hydrophilic, biocompatible, biodegradable, and antibacterial. Chitosan can act as a HP stabilizer by adsorbing transition metal ions such as removing free radicals that are released during the bleaching treatment (Jayakumar et al. 2011). Chitosan-doped TiO_2 nanoparticle bleaching agents can combine the photocatalytic property of TiO_2 with the adsorption property of chitosan, therefore making it appropriate in order to bleaching processes (Podust et al. 2014).

Surmelioglu et al. (2021b) demonstrated that a combination of chitosan-doped TiO_2 nanoparticles and 6% HP presents further effective tooth bleaching. Again, in a study by Surmelioglu et al. (2021a), it was determined that bleaching gels containing high concentrations (40% HP) did not cause systemic genotoxic damage.

In the study evaluating the toxicity of chitosan on cells, Fernandes et al. (2008) showed that chitosan exerted toxic effect onto human red blood cell line based on the concentration (>0.1 mg/mL). Wiegand et al. (2010) demonstrated that chitosan induced apoptosis in human keratinocyte cell lines depending on duration and concentration. It was thought that this might be related to release of anti-inflammatory cytokines off chitosan and induction of apoptosis-related caspases

(Wiegand et al. 2010). In a by research by Ugur Aydin et al. (2018), chitosan demonstrated toxic effects onto human gingival cell line depending on exposure period. Contrary to these studies, Chellat et al. (2000) stated that chitosan did not demonstrate toxicity onto fibroblast cell line. Difference in studies was most likely due to methodological differences such as concentration, cell line, and test method.

Ozone

Ozone (O_3), which consists of three oxygen atoms, is the third strongest oxidant after fluorine and persulfate (Bocci 2006; Burns 1997). Ozone has analgesic and antimicrobial properties which are extremely powerful against bacteria and viruses. Also, it can induce blood circulation and immune system response (Bocci 2006; Burns 1997).

A study showed that using ozone to improve the effectiveness of 8% CP did not present significant results (Manton et al. 2008). Nevertheless, in another study, the ozone applied agent conducted a superior outcome about bleaching effect, which also decreased the surface roughness of composite resin comparing the 30% CP (Elhamid and Mosallam 2010). Grundlingh et al. (2012) found that ozone presents a similar efficacy as 45% CP.

There might be various reasons for better bleaching efficacy obtained when ozone is combined with HP. The chief among these is that ozone provides constant $O_2^{-\bullet}$ release compared with HP. Also, HP decomposes very slowly, and there has a limitation for the fast reaction due to being a weaker oxidant than ozone. Besides that, ozone is a stable molecule (Al-Omiri et al. 2016).

Low-Intensity Laser

Although the results are not conclusive, the use of anti-inflammatory agents and low-level laser therapy can reduce pulp damage after teeth whitening treatment (Benetti et al. 2018; Vargas et al. 2014). According to Moshonov et al. (2001), in a clinical practice, a low-energy laser should be preferred to achieve more positive results in terms of whitening. It was thought that low-dose laser therapy applied can have the potential to minimize pulp damage occurred with bleaching (Carminatti et al. 2020; Dantas et al. 2010). Although low-dose laser therapy is recommended for clinical use, a sufficient and safe protocol in order to use laser therapy has not been determined yet. While the protection produced by a less enamel-dentin thickness is less effective in preventing the heating caused by light within animals, the thicker enamel-dentin structure requires a larger wavelength of light at low-intensity laser therapy in order for the treatment to achieve the target tissue effectively (Buchalla and Attin 2007).

In a research by Dantas et al. (2010), 35% HP was applied on the dental pulp fibroblasts in the 96-well plate for 40 min, and then phototherapy was performed using a low-intensity laser (diode laser) at two different wavelengths (660 and 780 nm), with an output power of 40 mW. The substances released during the bleaching were added to the medium used in research. The cell viability was

measured in human pulp fibroblast cells before irritation, and it was found that the substances released in the bleaching application had a cytotoxic effect. Although the substrates were found to reduce cell viability, it has been reported that phototherapy with a low-intensity laser can compensate to the cytotoxic effects of the gel.

Applications to Prognosis

Inflammation is an acute or chronic response of the human body which occurs as a result of any stimulus. The inflammation, which is closely related to the oxidative stress, causes a strong metabolic response in many different diseases. In addition, chronic inflammation promotes increased production of free radical oxidants. Agents used in bleaching treatment such as HP and CP may enhance the inflammatory cells, migration of macrophage cells, some necrotic areas, clogged large-caliber blood vessels, and temporary collagen breakdown within the pulp chamber during bleaching (Vaz et al. 2016). These collagen deteriorations can be transitory due to the self-repairing property of the pulp. Cytokines that contribute to the formation of ROSs by being released during inflammation are interleukin-1 and interleukin-6 (IL-1, IL-6), tumor necrosis factor-α (TNF-α), and interferon-γ (IFNγ) (Chapple 1997). In addition, while IL-1β are upregulated with caries lesion and pathogens, tumor necrosis factor β (TNF-β) cytokines are also generated there in irreversible dental pulpitis (Renard et al. 2016). One of these cytokines, IL-6, is a central mediator of inflammatory procedure. In addition, IL-6 is considered a biomarker of oxidative stress, physiological aging, and frailty (Fasshauer and Paschke 2003). Although there is a potent association among IL-6 levels and single-stranded breaks of DNA in healthy aging with exercise (Broedbaek et al. 2011), there is no association between IL-6 levels and oxidative damage indicators of RNA within aging communities with low-grade inflammation (Broedbaek et al. 2009).

Damage Biomarkers

Electron Paramagnetic Resonance

Kawamoto and Tsujimoto (2004) reported that glycine did not cause any change in the OH^- signal; however, proline and alanine caused a decrease in the OH^- signal using electron paramagnetic resonance (EPR). It was thought that proline and alanine were more sensible to attack by the OH^-. In addition, as a result of the loss of the OH^- signal, the structures of these proteins changed, but glycine was excluded from this reaction (Kawamoto and Tsujimoto 2004). Moseley et al. (1998) reported that bovine alveolar bone proteoglycans exposed to HP or OH^- for 24 h caused a change in amino acid composition. When HP concentrations increased after 24 hours, proline and alanine levels remained unchanged, although there was a significant enrichment in glycine relative to detectable total amino acids.

Determination of Oxidative Stress Markers

Determination of Lipid Peroxidation Products

ROS may produce detriment to several biomacromolecules such as DNA, proteins, and lipids through cell death. One of the harmful effects of ROS is that it causes peroxidation of membrane lipids. The entity of carbon-carbon double bonds within polyunsaturated fatty acids that composed the structure of the membrane makes itself vulnerable to damage due to ROS (Ghosh et al. 2015). Lipid peroxidation may cause deterioration of the structure of cell membranes. In addition, it can inactivate the membrane-bound proteins (Birben et al. 2012). Al-Basher et al. (2019) found a significant enhancement within levels of malondialdehyde (MDA), one of the lipid peroxidation markers, in the liver, kidney, and heart of rats after oral administration of 35% CP. MDA is widely preferred as a sensitive assay method to use within animal tissues for lipid peroxidation (Patton and Kurtz 1951). Determination of MDA levels is decent measurement method of peroxidation, which is known as one of the primary mechanisms of cell damage leading to necrosis or apoptosis (De Ferreyra et al. 1989).

Determination of Protein Damage (Hyaluronic Acid and Proteoglycans)

Proteoglycan masses of articular cartilage are constituted with proteoglycan subunits in non-covalent unions with hyaluronic acid. It has been recommended that reactive oxygen metabolites can take part in the breakdown of connective tissue by excessive accumulation of transition metal ions in joints under inflammatory arthritic conditions (Halliwell and Gutteridge 1984, 1985). Oxygen metabolites of partially reduced production are an outcome of regular aerobic metabolism (Chance et al. 1979). It has been shown that these metabolites can be activated or inactivated proteinases and inactivate proteinase inhibitors (Vissers and Winterbourn 1987) in particular circumstances. Previous studies demonstrated that reactive oxygen metabolites reduce the proteoglycans directly (Bartold et al. 1984; Bates et al. 1984) and reported that HP treatment aggregates proteoglycan in neonatal human articular cartilage (Roberts et al. 1987).

DNA Damage

Although HP is among the components of living cells, it becomes hazardous to health when exogenous HP levels exceed cytological protection levels (Tredwin et al. 2006). It has been demonstrated that the enhancement of HP above the cytological limit takes part in the development of cancer by causing DNA damage, mutations, and genetic imbalance (López-Lázaro 2007). Stress caused by oxidative by-products released as a result of the metabolism of HP may contribute to many pathological conditions and diseases, including cancer (Hassell 1993).

When OH^-, the most prominent oxygen-free radical that damages the basic biological structures, is generated adjoining to cellular and mitochondrial DNA, it attacks the DNA strands and causes the addition of new radicals to the nucleobases contained therein, resulting in the formation of various oxidation products (Valko et al. 2004). It is known that the OH^- radical forms C8-hydroxyguanine (8-OHGua)

or its nucleoside forms deoxyguanosine (8-hydroxy-2′-deoxyguanosine) due to its interaction with guanine, one of the nucleobases of the DNA chain. The OH^- reaction causes the formation of radical adducts, followed by removal of one electron to form 8-hydroxy-2′-deoxyguanosine (8-OH-dG) (Valavanidis et al. 2009). The assignment and analysis of 8-OHdG, utilized as biomarker for oxidative stress, aging, and carcinogenesis, may be performed with animal or human samples such as urine and leukocyte DNA (Collins 2000).

In a research performed by Del Real Garcia et al. (2019) on 113 individuals using a bleaching strip containing 10% HP, 60 individuals used the bleaching strips for 10 days and the remaining people were determined as the control group, and oxidative DNA damage was tested by ELISA. For oxidative DNA damage, results were obtained by measuring 8-OHdG from the saliva of individuals exposed and unexposed onto the bleaching strips which show that exposed individuals present higher levels at different sampling durations compared to basal levels of 8-OHdG.

Measurement of the Antioxidant Defense System

Antioxidant Enzymes

Biomarkers of different metabolic systems, including antioxidant enzymes, are widely used to monitor physiological structure with precision. There are many different antioxidant enzymes that are used as biomarkers in animals such as CAT, SOD, glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione S-transferases (GST) (Canli et al. 2019). These biomarkers provide the elimination of oxidants released as a result of the metabolism of xenobiotics, but if changes occur in enzyme activities, this may lead to oxidative stress. Of these biomarkers, CAT converts HP into the water, while SOD converts the $O_2^{-\bullet}$ to HP, which are also known as the first enzymes in the line of defense to fight oxidants. While GST accelerates glutathione and xenobiotic conjugation, GPx removes toxic substances due to hydrogen and organic peroxides. Finally, the last of these enzymes, GR, is used in antioxidant defense by reducing oxidized glutathione (Atli et al. 2016).

Determination of Total Antioxidant Activity

The total antioxidant capacity can be determined with the aid of a test solution. It takes part in determining the antioxidant capacity of living tissues by measuring the number of scavenged free radicals (Bartosz 2010; Pinchuk et al. 2012). Among these tests, the most widely used is Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) equivalent antioxidant capacity, which depends on the time used for measurement and the radical-based modifications formed (Erel 2004). In addition to direct tests such as the oxygen radical absorbance capacity (Cao et al. 1993), indirect tests such as the ferric decreasing potentiality of plasma (Benzie and Strain 1996) and the cupric decreasing antioxidant capability (Apak et al. 2005) are also included.

Total antioxidant capacity tests are divided into two as procedures based on hydrogen atom transmission or procedures based on single-electron transmission. Methods based on hydrogen atom transfer are rapid and pH-independent and

calculate the antioxidant's ability to eliminate the free radicals through hydrogen donation (i.e., oxygen radical absorbance capability). Methods based on single-electron transfer, which are pH-dependent, detect the ability of a potent antioxidant to transfer an electron to reduce any compound (i.e., ferric reducing the quality of plasma, cupric decreasing antioxidant capability, and Trolox equivalent antioxidant capability) (Bartosz 2010).

Measurement of Low-Molecular-Weight Antioxidants

Antioxidants may be examined in two main groups, namely, antioxidant enzymes and low-molecular-weight antioxidants (LMWA) (Kohen et al. 1997). LMWA structure is a major group including more than a few hundred complexes unlike the antioxidant enzymes consisting less amount of proteins. Since these low-weight molecules have a hydrophilic or lipophilic structure, they can simply penetrate into the cell through pores within the membrane and also be concentrated in high concentrations where oxidative damage is known to occur. They have a wide range of activity against ROS and have the ability to be reproduced by the cell. Although it is obtained from a wide variety of resources such as endogenous biosynthesis, and waste products of the living cell, it is primarily of dietary origin (Rice-Evans and Miller 1994; Whitehead et al. 1992). Compounds such as glutathione, ascorbic acid, α-tocopherol, and cysteine, the most well known of these LMWA, maintain the organism toward free radical detriment (Langemann et al. 1992).

Vitamin E, which takes part in prevention of oxidative events, consists of a blend of tocopherols and tocotrienols (α, β, γ, and δ) (Aggarwal et al. 2010). Antioxidant effect of vitamin E is provided by the α-tocopherol isomer, which can incorporate the cell membrane and stop the spread of lipid peroxidation (Upadhyay and Misra 2009). Previous studies (Guney et al. 2011; Makpol et al. 2010) demonstrated the protective effects of α-tocopherol such as prevention of oxidative stress propagation, cell membrane stabilization, increase of cell viability, and enhanced number of endogenous antioxidants. In a study by Vargas et al. (2014), a reduction within HP-mediated aggression to cells was observed for all α-tocopherol concentrations used after HP.

Measurement of Enzyme Cofactors

SODs, which constitute the initial defense line of the cell defense system toward ROS, and protect organisms from its toxic effects per catalyzing the reduction of O_2^- toward HP and O_2, are metalloproteins and can minimize the detrimental effects that may occur (Apel and Hirt 2004; Fridovich 1995; Miao and Clair 2009). These SODs are variously classified due to the metal cofactors required about the O_2^- scavenging function such as copper-zinc SODs (Cu-Zn SODs), iron SODs (Fe SOD), manganese SODs (MMA-SOD), and nickel SODs (Ni SOD). When mammalian cells were examined, it was observed that Cu-Zn SODs were found inside the cytoplasm (referred to as SOD1) and/or extracellular areas (SOD3 or EC-SOD), but Mn-SODs (referred to as SOD2) were located within the mitochondria (Zelko

Table 1 Bleaching agents and the supplementary contents

Peroxide	Nonperoxide	Assit contents
Hydrogen peroxide	Papain,	Polymeric nanoparticle
Carbamide peroxide	Bromelain	Nitrogen-doped titanium dioxide
	Ficin	Nano-bioactive material
	Chlorine dioxide	Chitosan
	Sodium chloride	Ozone
	Sodium bicarbonate	Low-intensity laser

et al. 2002). Although HP can inactivate Cu-Zn SODs, and Fe SODs, it does not affect the Mn-SOD (Kang et al. 2014) (Table 1).

Mini-dictionary of Terms

Bleaching agents are used to lighten tooth surfaces.

Free radicals are atoms which include an unpaired electron.

Biomarker is a marker of regular biological and/or pathogenic procedures, or pharmacological responses to therapeutic intervention.

ROS is derived from HPs which react with chromogens to break the double bond of organic structures, reducing tooth coloration.

Oxidative stress is a phenomenon produced by an unbalance among production of ROS and free radicals and ability of antioxidant capability of biological structure.

Key Facts of Bleaching Agents as Toxic Compounds and Biomarkers of Damage

1. Agents used in bleaching treatment cause the generation of reactive oxygen metabolites.
2. Reactive oxygen metabolites which are released induce discoloration on buccal surface of the teeth to whiten by breaking down the carbon bonds.
3. However, these metabolites can generate many side effects such as DNA damage, genotoxicity, cytotoxicity, mineral loss, and tooth sensitivity.
4. In order to reduce these side effects, many different new methods have been developed, and newly developed agents have been produced instead of traditional bleaching agents.
5. There are limitless biomarker procedures to measuring the reactive oxygen metabolites. When deciding which method to use, attentive research should be done.

Summary Points

1. Prevention of the damage caused by ROSs generated by HP is achieved by numerous finely tuned mechanisms in the human body.

2. ROSs can cause many diseases such as DNA damage, genotoxicity, and cytotoxicity.
3. Biomarkers are very important in the primary diagnosis, prognosis, and treatment of diseases.
4. Many lipid, nucleic acid, or protein biomarkers can be used to measure oxidative stresses.
5. ROSs can be determined using the measurement of antioxidants as biomarkers.
6. In addition to these, enzyme cofactors can be used as biomarkers (Table 1).

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Endocrine Disruptors and Markers of Anthropogenic Activity: A Risk for Water Supply and Health

39

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Contents

Abstract

Numerous chemical substances are commonly used in everyday human activities. Some of these substances are considered micropollutants, with the potential to disrupt the endocrine system, threatening human and animal health. Among these substances, bisphenol A, phthalates, and ethinyl estradiol can reach the

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environment through the inadequate disposal of effluents or the inefficiency of conventional treatment plants. Therefore, this chapter describes the main concepts, examples, health effects, and methodologies for the extraction and analysis of endocrine disruptors in water.

Keywords

Micropollutants · Bisphenol A · Phthalates · Water resources · Water pollution · Water supply · Endocrine system · Analytical chemistry · Solid-phase extraction · Liquid chromatography

Abbreviations

ABRASCO	Brazilian Association of Collective Health
ANVISA	National Health Surveillance Agency
BBP	Butyl benzyl phthalate
BPA	Bisphenol A
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
CAS	Chemical Abstracts Service
CEC	Commission of the European Communities
DBP	Dibutyl phthalate
DCM	Dichloromethane
DEP	Diethyl phthalate
DEs	Endocrine disruptors
DMP	Dimethyl phthalate
E1	Estrone
E2	Estradiol/17β-estradiol
E3	Estriol
EDCs	Endocrine disruptor compounds
EE2	Ethinyl estradiol/17α-ethinyl estradiol
EFSA	European Food Safety Authority
ESI	Electrospray ionization
FAO	United Nations Food and Agriculture Organization
H_2O	Water
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
IT	Ion trap
LC	Liquid chromatography
LLE	Liquid-liquid extraction
MeOH	Methanol
MIP	Molecular imprint polymers
MISPE	Molecularly imprinted polymer solid-phase extraction
MS	Mass spectrometry
MSPDE	Matrix solid-phase dispersion extraction
MSPE	Magnetic solid-phase extraction
PAEs	Phthalic acid esters

PCBs	Polychlorinated biphenyls
PET	Polyethylene terephthalate
SLE	Solid-liquid extraction
SPE	Solid-phase extraction
SPME	Solid-phase microextraction
TDI	Tolerable daily intake
TMCS	Trimethylsilyl chloride
TOF	Time-of-flight
TQ	Triple quadruple
UHPLC	Ultrahigh performance liquid chromatography
UNICEF	United Nations Children's Fund
UPLC	Ultra-performance liquid chromatography
WHO	World Health Organization
WWF	World Wide Fund for Nature

Introduction

In recent decades, environmental quality has significantly decreased, mostly because of population increase, industrial and agricultural development, and natural resource exploitation. Environmental quality is the basis for preserving life for future generations, with water being the substance that sustains and allows the structuring of human society (Reis Filho et al. 2007). Nowadays, there is a global concern in view of the threats of pollution, unsustainable resource use, climate change, pressures associated with land use, and the risk of scarcity (Fernandes et al. 2011). Water contamination intensified after World War II due to the manufacturing and introduction of xenobiotic compounds into the aquatic environment, with negative effects on humans, animals, and aquatic ecosystems (Américo et al. 2012). The accumulation of these compounds in water resources poses a risk to human health (Cabrera et al. 2008).

The Joint Water Supply, Sanitation, and Hygiene Monitoring Program of the World Health Organization (WHO) and the United Nations Children's Fund (UNICEF) has estimated that in 2017, about 2.2 billion people (29%) did not have access to safe drinking water (UNICEF and WHO 2019). There is evidence that qualitative and quantitative changes in aquatic ecosystems in Brazil are related to anthropogenically produced compounds that have reached catchment water sources. This aspect, together with the disposal of raw sewage and the maintenance of outdated technologies in water and sewage treatment plants, highlights the need for public policies aimed at improving basic sanitation conditions and the development and implementation of new technologies to control the levels of micropollutants in drinking water (Sodré et al. 2007; Neto and Sarcinelli 2009; Marques et al. 2007).

Micropollutants are organic and inorganic compounds that, even at low concentrations (at μg L^{-1} and ng L^{-1} levels), present a potential risk to human health and the environment. They encompass a great diversity of natural or synthetic chemical

compounds, including drugs, personal care products, plasticizers, hormones, surfactants, flame retardants, pesticides, and nanoparticles (Cunha et al. 2016). Their physicochemical characteristics are dictated by their small size, large surface area, specific shape, and chemical composition. The increased production and use of nanomaterials has led to the need for more information on the potential impacts on human health and the environment (Farré et al. 2011). In this context, several environmental control agencies have identified a growing number of micropollutants that have endocrine-disrupting activity (Costa et al. 2014).

All physiological processes in the human body are governed by two systems: the nervous system and the endocrine system. The first controls physiological processes through nerve impulses conducted by neurons, whereas the second uses chemical messengers, called hormones, to mediate these processes. The set of cells responsible for the synthesis and excretion of hormones in the bloodstream is called the gland (Sodré et al. 2007). According to the World Health Organization (WHO), the endocrine system regulates the body's metabolism and functioning. Endocrine glands secrete hormones that act on their target organs. Thyroid hormones, for example, are essential for brain development and normal metabolism throughout the body (WHO 2012a). Thus, endocrine disruptors (EDs) are natural or synthetic chemicals responsible for causing changes in the functions of the endocrine system, with adverse effects on the individual's health. Some endocrine disruptors act as "hormone mimics," whereas others prevent natural hormones from carrying out their functions (Hormone Health Network 2021; Commission of the European Communities – CEC 2007). The latter are generally derived from pharmaceutical products and plastics and their waste present in sewage and industrial effluents. Disruptors can be found in surface water and groundwater, marine sediments, soil, biological sludge from effluent treatment plants, and even in drinking water. As they are continuously introduced into the environment in detectable concentrations, they can affect water quality, impacting public health (Bila and Dezotti 2007; de Souza 2011).

Effects of Endocrine Disruptors on Public Health and the Environment

Studies on the impacts of exposure to endocrine disruptors have identified a number of effects on growth, development, and reproduction in humans and wildlife over the past few years (Luckenbach et al. 2010). According to the World Health Organization, adverse effects of EDs on adipose tissue, adrenal glands, and the pancreas have been the main focus of research (WHO 2012a). For example, Sodré et al. (2007) were motivated by observations on the occurrence of abnormalities in the endocrine system of animals in the environment. Chronic alterations in the development and reproduction of several species have been attributed to the presence of chemical substances, mainly in natural aquatic systems.

The health issues resulting from the consumption of water contaminated with pesticides, for example, vary according to the active ingredients involved. Among the problems already identified and published in the literature, issues in the central

nervous, cardiovascular, and reproductive systems as well as an increased risk of developing cancer stand out (Neto and Sarcinelli 2009). According to the Brazilian Association of Collective Health (ABRASCO) (Carneiro et al. 2012), even if some of the active ingredients can be classified as moderately or slightly toxic, based on their acute effects, chronic effects can occur over months, years, or even decades after exposure, manifesting in various diseases such as cancers, congenital malformations, and endocrine, neurological, and mental disorders. In the case of humans, the embryonic stage is much more susceptible to the action of EDs compared to the adult individual (Sodré et al. 2007). The World Health Organization publication entitled "Possible developmental early effects of endocrine disrupters on child health" (WHO 2012a) presents the possible effects of endocrine disrupters on child health. The document also warns that some of the endocrine disruptors, such as polychlorinated biphenyls (PCBs), also have adverse effects on neurocognitive development. Pontelli et al. (2016) reported that several scientific studies suggest an association between endocrine disruptors and obesity in humans. According to the authors, there is an increase in the prevalence of obesity worldwide, which is considered a concern given its potential impact on the health system.

According to the World Health Organization (WHO 2012b), chemicals interfere with metabolism, fat storage, bone development, and the immune system. It is estimated that 1.5 billion adults worldwide are overweight or obese and that the number of people with type 2 diabetes increased from 153 million to 347 million between 1980 and 2008. Costa et al. (2014) investigated the effects of endocrine disruptors on the development of the female reproductive tract. According to the authors, EDs could explain the increase in the prevalence of some non-communicable diseases such as obesity, diabetes, thyroid diseases, and some types of cancer. The authors also emphasize that several endocrine disruptors, such as pesticides, bisphenol A, phthalates, dioxins, and phytoestrogens, can interact with the female reproductive system and lead to endocrine disruption. Cunha et al. (2016) highlighted that the synthetic estrogen 17α-ethinyl estradiol, the main component used in oral contraceptive formulations, is one of the main compounds responsible for causing adverse effects in the endocrine system of several species.

According to the Food and Agriculture Organization of the United Nations (FAO), a decrease in aquatic species is observed, mainly due to the increase in pollution by pesticides that threaten ecosystems and biodiversity (FAO 2002).

Luckenbach et al. (2010) studied the potential effects of endocrine-disrupting compounds on bivalve populations in the Chesapeake Bay, USA. Fewer studies have addressed the effects of exposure of bivalve mollusks to endocrine disruptors, but there is evidence for several species that a number of these compounds can affect sex determination, gonadal and gamete development, and egg and sperm viability and function as well as larval development. Intergenerational effects, in which exposure of one generation to DEs reduces larvae survival in the next generation, have also been observed.

The document "State of the Science of Endocrine-Disrupting Chemicals 2012" (WHO 2012b), prepared by a group of experts from the United Nations Environment Program and the World Health Organization, elucidates that the main concerns

regarding endocrine disruptors, such as diseases and disorders related to the endocrine system, thyroid-associated neurobehavioral disorders, and global cancer rates (breast, endometrium, ovary, prostate, testicular and thyroid), have increased over the past 40–50 years; the prevalence of obesity and type 2 diabetes has increased in the last 40 years.

Examples of Endocrine Disruptors and Markers of Anthropogenic Activities

The use of chemical substances from different sources is gradually increasing. Such substances can bioaccumulate in various organisms and remain in the environment for long periods of time (Vettorello et al. 2017). About 800 chemicals are interfering or suspected of interfering with the endocrine system. However, only a small portion of chemical compounds have been investigated in tests and their endocrine effects on organisms identified (WHO 2012b).

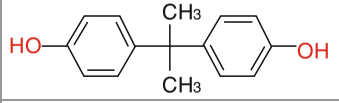
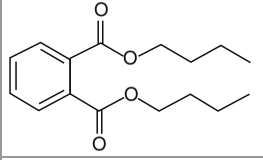
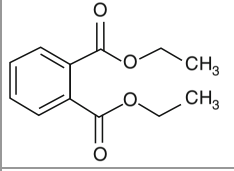
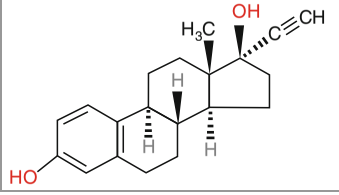
Among the compounds that can negatively impact the hormonal system are substances with plasticizing properties that are widely used in polymeric materials to provide flexibility (Souza et al. 2012). It is noteworthy that the large production of polymers in Brazil ranks fourth among the countries that generate the most plastic waste in the world, with around 11.3 million tons/year (World-Wide Fund for Nature – WWF 2019).

There are several chemical compounds that fit these descriptions and are classified as endocrine disruptors, which can be either synthetic substances, such as phthalates, the plasticizer bisphenol A (BPA), and the hormone ethinyl estradiol (EE2) and its by-products estrone (E1) and estriol (E3), or natural substances, such as estrogens and the hormone estradiol (E2) (Bila and Dezotti 2007; Lima et al. 2017). Table 1 shows some examples of these compounds and their main characteristics.

Bisphenol A (BPA) is one of the largest chemical compounds and is used in the production of plastics that cover the inner surface of food cans, polycarbonate compounds, and epoxy resin. After polymerization, some BPA molecules remain free and can contaminate the food and beverages in these containers (Costa et al. 2014; Alammari et al. 2020). In addition to being a plasticizer commonly found in the environment, this compound is associated with several health issues such as cancer, infertility, obesity, cardiovascular disease, diabetes, asthma, and neurodegenerative diseases (Catenza et al. 2021; Moon et al. 2020; Lee et al. 2021; Mendy et al. 2019; Gupta et al. 2020).

Phthalic acid esters (PAEs), or phthalates, are synthetic substances used as additives and plasticizers; they are widely applied in cosmetic products, carpets, pharmaceutical pills, and plastic materials to modify flexibility and durability. Because PAE molecules are only additives in these polymers, they manage to migrate from the packaging to the food product, making them common pollutants in the environment (Dobaradaran et al. 2020; Salazar-Beltrán et al. 2018). Within the group of phthalates, there is dibutyl phthalate (DBP), which is also present in

Table 1 Examples of endocrine-disrupting micropollutants and their respective CAS number, molecular structure and formula, molar mass, and application

Type	Name	CAS-no.	Molecular structure	Molecular formula	Molar mass (g/mol)	Application
Plasticizers	**Bisphenol A**	80-05-7		$C_{15}H_{16}O_2$	228.29	Used in the production of polycarbonate and epoxy varnishes
	Dibutyl phthalate	84-74-2		$C_{16}H_{22}O_4$	278.34	Additive to adhesives and printing inks; ectoparasiticide; cosmetic (enamels)
	Diethyl phthalate	84-66-2		$C_{12}H_{14}O_4$	222.24	Used in cosmetics as a solvent for perfumes; detergent base and aerosol sprays; present in the composition of medicines
Hormone	**Ethinyl estradiol**	57-63-6		$C_{20}H_{24}O_2$	296.41	Semi-synthetic estrogen used as a component of oral contraceptives

Source: Prepared by the authors (ANVISA 2017; Chemspider 2017; PubChem 2017)

materials such as food containers, toys, medical devices, cosmetics, and other personal care products (Deng et al. 2018). It can cause fertility problems in both men and women, and studies have shown that patients with polycystic ovary syndrome had significantly higher levels of diethyl phthalate (DEP) and BPD than those in the control group (Tu et al. 2019; Xu et al. 2011).

Diethyl phthalate (DEP) is a short-chain, low-molecular-weight phthalate, like DBP, and is found in personal care products, medications, certain dietary supplements, adhesives, and printing inks (Sakhi et al. 2014). This compound has been associated with adverse effects on the reproductive process, including miscarriage, premature birth, and problems with pubertal development (Radke et al. 2019). In addition to these effects, a study conducted in Taiwan by Wang et al. (2020) showed positive results associating the level of DEP with the decrease in the values of pulmonary function tests.

In the estrogen class, there is 17α-ethinyl estradiol (EE2), which is a compound commonly used in oral contraceptives and medicines. It is a synthetic derivative of 17β-estradiol (E2), which is a natural hormone with high estrogenic activity. The EE2 is more resistant to biodegradation when compared to E2, although the structure is similar; EE2 therefore accumulates more easily in the environment (Vieira et al. 2020). It is one of the most potent environmental endocrine-disrupting compounds (EDCs), causing decreased fertility and fecundity as well as feminization in fish (Voisin et al. 2018; Aris et al. 2014).

Due to the adverse effects of to the endocrine disruptors currently used, the European Food Safety Authority (EFSA) has specified the tolerable daily intake (TDI) values for some of these compounds, such as DBP and BPA, which are 50 and 4 μg/kg/body weight/day, respectively (EFSA 2015, 2019).

Extraction Methods for Endocrine-Disrupting Micropollutants

The investigation of micropollutants in environmental matrices involves various sample preparation steps to improve the sensitivity of the analytical methodology. The term extraction is the separation of one or more components of a mixture using appropriate solvents (Priego-Capote and Castro 2004). For analysis of a sample by liquid chromatography, for example, it is usually necessary to perform extraction and purification prior to the procedure. There are several reasons for using these techniques, among them the fact that the analytes are present in trace level concentrations, thus requiring sample selectivity. As there is no universal procedure for extracting analytes, the methodology must be evaluated so that the extraction method is efficient and can extract the compounds present in the sample in a quantitative way without interconverting them and to ensure the compatibility of the extracting medium (solvent) with the separation and detection technique (Moreira 2013).

There are two main strands for the extraction methods, the solid-liquid (SLE) and the liquid-liquid (LLE) technique, with SLE being the most applied and inserted in this medium and the solid-phase extraction (SPE) method the most used of that

strand. The SPE is one of the most applied techniques in the analysis of endocrine disruptors and is used to carry out analyte pre-concentration and purification, minimizing the matrix effect of the sample, in addition to being a versatile technique due to the availability of several solvents, facilitating the extraction of a great range of compounds (Salgueiro-González et al. 2017; Fontanals et al. 2019). Solid-phase extraction uses a liquid or gas sample in contact with a solid phase or sorbent, where the analyte is selectively adsorbed onto the solid-phase surface. This phase in turn is then separated from the solution, and other solvents (liquids or gases) are added. The first solvent used removes possible adsorbed matrix components; eventually, an elution solvent is brought into contact with the sorbent to selectively desorb the analyte (Dean 1998; Quinete 2005).

This technique has several variations that are applied according to the affinity of the method with the compound to be extracted and its origin, such as solid-phase microextraction (SPME), magnetic SPE (MSPE), SPE with molecular printing (MISPE), and solid-phase matrix dispersion extraction (MSPDE) (Deng et al. 2019; Tasmia and Jan 2020; Li et al. 2018; Tsalbouris et al. 2021; Soares et al. 2021). In its various applications and methodologies, solid-phase extraction is often used together with high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS).

Endocrine Disruptor Analysis

Among the available instrumental techniques for the quantification of organic micropollutants in environmental samples, chromatography is, without doubt, the main one. High-performance liquid chromatography (HPLC) is one of the main techniques used in the analysis of nonvolatile and/or thermally unstable compounds (Lanças 2013). Column liquid chromatography is a technique widely used for the isolation of natural products and the purification of chemical reaction products. The most used stationary phases are silica and alumina; however, these adsorbents can simply serve as support for a liquid stationary phase. Solid stationary phases lead to separation by adsorption and liquid stationary phases by partition. The use of solvents must be continuous, and the different components of the mixture move at different speeds depending on their relative affinity for the adsorbent and for the eluent. Thus, the ability of a given eluent to carry an adsorbed compound onto the column depends on the polarity of the solvent with respect to the sample (Degani et al. 1998).

Despite being an excellent separation technique, HPLC requires a confirmatory technique when qualitative analysis (confirmation of chemical identity) is also required. Among the various options currently available, mass spectrometry (MS) is the technique of choice to provide necessary structural information. The coupling between these two techniques gives rise to a versatile analytical tool with great potential in qualitative and quantitative analysis: LC/MS (liquid chromatography coupled with mass spectrometry) (Lanças 2013). High-performance liquid chromatography coupled with mass spectrometry is used for the identification,

quantification, and separation of different compounds in a mixture (Rathore and Joshi 2018). The applicability of the association between these two techniques facilitated several studies in the area of endocrine disruptors, such as the study by Alammari, Khan, and Aqel (2020), who investigated the presence of BPA in water samples sold in bottles of polyethylene terephthalate (PET) and glass. To determine the concentrations in these samples, the authors used an improved method based on SPE, employing the SPE C18 cartridge. For identification, ultra-performance liquid chromatography (UPLC-MS/MS) was used, obtaining recoveries between 96.81% and 99.77%.

In another study conducted by Goeury et al. (2019), the authors sought to verify the presence of BPA and 13 other hormones in tap water, surface water, effluent, and wastewater; the analyzed compounds were testosterone, progesterone, medroxyprogesterone, levonorgestrel, enorethindrone, androstenedione, estrone, β-estradiol, α-estradiol, equilin, equilenin, ethinyl estradiol, and estriol. For this, they applied an alternative methodology that met the requirements of Method 539.1 of the US Environmental Protection Agency, applying SPE coupled online to ultrahigh performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS), obtaining efficiencies between 64 and 79% and coefficients of determination (R^2) >0.9980. The research by Avar et al. (2016) proceeded with the extraction, conditioning SPE cartridges with 15 mL of methanol and balanced with 20 mL of water. Subsequently, 1000 mL of the sample was loaded, and the cartridge was washed with 20 mL of water and eluted with 15 mL of methanol, followed by concentrating the sample using a rotary evaporator at 35 °C, dissolving it in methanol (3 × 200 μL) and transferring it to Eppendorf tubes.

Another study by Barciela-Alonso et al. (2017) sought to develop a method for the determination of phthalates, namely, dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), diethyl phthalate (DEP), and dimethyl phthalate (DMP) in water samples and wine. For this, the pre-concentration process with column SPE was carried out, filling a glass syringe with 200 mg of molecular imprint polymer absorbers (MIP). Conditioning was carried out using 15 mL of methanol and 15 mL of ultrapure water, followed by 25 mL of water sample at a flow rate of 1 mL min^{-1}. Subsequently, the cartridge was washed with 1 mL of acetonitrile/methanol (1:1) and eluted with 4 mL of methanol. The treated samples were analyzed by liquid chromatography and mass spectrometry (HPLC-ESI-MS), resulting in a recovery of 94–96%. Otomo (2015) determined the presence of endocrine disruptors and markers of human activities in water samples from the Guarapiranga Reservoir, which supplies the metropolitan region of São Paulo. In the applied methodology, the samples used were acidified with hydrochloric acid (HCl) diluted 1:1 (v/v) until pH 3. In the SPE process, the cartridges were conditioned with 5 mL of methanol (MeOH) and 5 mL of ultrapure MeOH/H_2O (pH 3) (1:9 v/v). A volume of 1 L of the sample was passed at a flow of 6 mL min^{-1}, and after the process was completed, the cartridges were washed with 5 mL of ultrapure MeOH/H_2O (1:9 v/v); finally, the samples were centrifuged for 20 min at 2500 rounds/min. Similarly, in the study by Otomo (2015), the elution process was carried out with 5 mL of dichloromethane and methanol (DCM/MeOH) (6:4 v/v) twice, in sequence,

and the liquid was transferred to a vial and evaporated with nitrogen. To the vial, 100 μL of derivatizer N,O-Bis(trimethylsilyl)trifluoroacetamide/trimethylsilyl chloride (BSTFA/TMCS) was added, and the mixture was heated at 100 °C for 30 min. After cooling, the volume was increased to 1 mL with DCM. Chromatographic analyses indicated the presence of bisphenol A (1061 ng L^{-1}) and dibutyl phthalate (12,921 ng L^{-1}). In the study by Machado et al. (2016), contaminants were evaluated in samples of raw and drinking water from the most populated regions of Brazil. The compounds were determined via solid-phase extraction, followed by analysis via liquid chromatography and mass spectrometry. Caffeine, triclosan, atrazine, phenolphthalein, and bisphenol A were found in at least one of the collected samples. Caffeine and atrazine were the substances most frequently detected in both drinking water and source. The caffeine concentrations in drinking water ranged from 1.8 ng L^{-1} to values above 2.0 μg L^{-1}, whereas the water sources showed concentrations from 40 ng L^{-1} to about 19 μg L^{-1}. For atrazine, the concentrations ranged from 2.0 to 6.0 ng L^{-1}. Maynard et al. (2019) investigated the occurrence of several chemical substances used in daily activities in a water supply system in the rural area of northeastern Brazil. Samples of raw water (underground and surface) and treated water (distribution network and reservoir) were collected, and the results showed that 50% of the samples contained caffeine, followed by cholesterol (45.5%), diethyl phthalate (31.81%), dibutyl phthalate (31.81%), bisphenol A (18.18%), and estradiol (4.54%). Table 2 provides a summary of examples of analytical methodologies for identifying endocrine-disrupting micropollutants in different matrices.

Final considerations: Endocrine-disrupting compounds are ubiquitous in the environment and pose a risk to human and animal health. In this regard, the need for studies that systematically assess their effects on health is highlighted. In addition, regulations of the maximum allowed limits, at a global level, must be expanded to ensure greater control. On the other hand, the reduction of the use of these compounds should be encouraged.

Applications to Prognosis

In this chapter, it was possible to review works that investigate the presence of endocrine-disrupting compounds in water and supply sources and their effects and analytical methodologies. Considering that endocrine disruptors are natural or synthetic chemicals, responsible for causing changes in the functions of the endocrine system (Hormone Health Network 2021; Commission of the European Communities – CEC 2007), some examples of adverse effects can be mentioned, as in neurocognitive development (WHO 2012a), bone development, immune system (WHO 2012b), and obesity (Pontelli et al. 2016), among others. Among the endocrine disruptors, bisphenol A (BPA) is one of the world's largest chemical compounds, used in the production of plastics that cover the inner surface of food cans (Costa et al. 2014; Alammari et al. 2020). In addition to being a plasticizer commonly found in the environment, this compound is associated with several health problems such as cancer, infertility, obesity, cardiovascular disease, diabetes,

Table 2 Summary of analytical methodologies for identifying endocrine-disrupting micro-pollutants in water and other matrices

Sample	Compounds	Technique and analysis	Source
Surface water	Bisphenol A, ethinyl estradiol, diethyl phthalate	LC-ION TRAP TOF Injection volume, 5 μL; flow, 0.2 mL min^{-1} Column, C18; run time, 35 min; oven temperature, 40 °C Mobile phase: water (a) and methanol (b) Source: ESI Temperature, 200 °C; dry gas, 100 kPa Nebulizer gas: 1.5 L min^{-1}	Moreira et al. (2011)
Drinking water, soft drinks, powdered milk	Phthalates and bisphenol A, etc.	TQ-ESI-MS Injection volume, 25 μL; column, C8 Temperature: 50 °C Temperature: 350 °C Gas flow: 10 mL min^{-1}	Khedr (2013)
Surface water	17β-Estradiol, 17α-ethinyl estradiol	LC-MS/MS Flow: 0.4 mL min^{-1} Column: C18 Run time, 10–15 min; temperature, 30 °C Mobile phase: formic acid in water (a) formic acid in acetonitrile (b)	Avar et al. (2016)
Surface water	17β-Estradiol, 17α-ethinyl estradiol and 4-noniphenol	LC/MS-ESI-TOF Injection volume, 5 μL; flow, 0.2 mL min^{-1} Column: C18 Mobile phase: methanol and water	Moreira et al. (2009)
Water, sediment, and biota	17β-Estradiol, estrone, bisphenol-a; 17α-ethinyl estradiol, etc.	HPLC-ESI-MS Flow: 500 μL Column: C18	Pojana et al. (2007)
Water in bottles containing polyethylene terephthalate (PET)	Bisphenol A	UPLC-MS/MS Column: C18 reverse phase Mobile phase: methanol and water Injection volume: 5 μL Source: ESI	Alammari et al. (2020)
Tap water, superficial and residual	Environmental Protection Agency priority endocrine-disrupting hormones and bisphenol A	LC-MS/MS Mobile phase: water and methanol Run time: 15.5 min	Goeury et al. (2019)

(continued)

Table 2 (continued)

Sample	Compounds	Technique and analysis	Source
Surface estuarine water	Multi-classes of endocrine-disrupting compounds	LC-MS/MS Column: C18 Mobile phases: 0.2% acetic acid in ultrapure water and methanol Flow: 0.30 and 0.35 mL min^{-1} Injection volume: 10 mL	Ismail et al. (2019)

Source: Prepared by the authors

asthma, and neurodegenerative diseases (Catenza et al. 2021; Moon et al. 2020; Lee et al. 2021; Mendy et al. 2019; Gupta et al. 2020). It is also noteworthy that for the determination of compounds such as bisphenol A in water, for example, the use of liquid chromatography coupled with mass spectrometry is widely used (Moreira et al. 2011; Khedr 2013; Avar et al. 2016; Moreira et al. 2009; Alammari et al. 2020; Goeury et al. 2019; Ismail et al. 2019).

Mini-dictionary of Terms

- Endocrine disruptors: Natural or synthetic chemicals responsible for causing changes in the endocrine system, causing adverse health effects.
- Micropollutants: Organic and inorganic compounds that, even at low concentrations, pose a potential risk to human health and the environment.
- Chromatography: Analytical technique of the separation of complex mixtures for compound identification.
- Water supply system: This encompasses the collection, adduction, treatment, and distribution of drinking water to serve a given population.
- Phthalates: Synthetic substances used to modify the flexibility and durability of products.

Key Facts of Endocrine Disruptors and Markers of Anthropogenic Activity: A Risk for Water Supply and Health

- More than two billion people do not have access to clean water.
- There are more than 800 substances with the potential to disrupt the endocrine system.
- Conventional treatment plants do not remove endocrine-disrupting micropollutants from water.
- Endocrine disruptors can cause decreased fertility, obesity, and diabetes, among others.

- The analytical techniques of solid-phase extraction and liquid chromatography are suitable for the determination of these compounds in water.

Summary Points

- Several micropollutants can be considered endocrine disruptors.
- Endocrine disruptors reach the environment through the inadequate disposal of effluents or the inefficiency of treatment systems.
- Endocrine disruptors can be found in water supply systems.
- Solid-phase extraction and liquid chromatography analysis coupled with mass spectrometry have been used to determine endocrine disruptors in water.
- More studies that assess the impacts of endocrine disruptors on human health and the environment should be encouraged.
- Preventive measures for the large-scale use of these compounds are necessary.

Cross-References

► LC-MS-Based Metabolomics in the Identification of Biomarkers Pertaining to Drug Toxicity: A New Narrative
► Lead and Aquatic Ecosystems, Biomarkers, and Implications for Humankind
► Soluble Guanylyl Cyclase Alpha1 Subunit as a Biomarker of Toxicity: Applications to Investigate Endocrine-Disrupting Chemicals

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Biomarkers in the Biomonitoring of Fluoride Toxicity: An Overview

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Contents

Abstract

Fluoride (F) is of great importance in dental science, particularly to control the disease that mostly affects the oral cavity: caries. However, some detrimental effects have been observed since the nineteenth century due to F exposure beyond safe limits. Several studies have investigated the relation between nontherapeutic exposure to environmental F sources and complications in mineralized and non-mineralized tissues such as the central nervous system, liver, kidneys, and digestive system. Preventive medicine has used advanced, noninvasive, and easy-to-obtain biomonitoring technologies to early diagnose overexposure to several

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toxicants such as fluoride in at-risk populations such as those living in areas with endemic fluorosis. However, the choice of the most appropriate biomarker depends on whether the F exposure is acute or chronic and must take into account factors such as age, target population, study aim, and potential exposure sources. Therefore, this chapter compilates the most recent and fundamental scientific evidence to better understand F biomonitoring, main sources of exposure, advantages, and complications, in addition to encouraging further research and highlighting damages caused by excessive F exposure.

Keywords

Fluoride · Biomarkers · Fluorosis · Biofluids · Biomonitoring · Review

Abbreviations

$Ca_{10}(PO_4)^6(OH)_2$	Hydroxyapatite
$Ca_5\ (PO_4)\ _3F$	Fluorapatite
CaF_2	Calcium fluoride
CAT	Catalase
DNA	Deoxyribonucleic acid
ERK	Extracellular signal-regulated kinase
F	Fluoride
GSH	Reduced glutathione
H_2SiF_6	Fluorosilicic acid
HMDS	Hexamethyldisiloxane
JNK	c-Jun N-terminal kinases
Na_2SiF_6	Sodium fluosilicate
NaF	Sodium fluoride
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B
NO	Nitric oxide
RT-qPCR	Quantitative reverse transcription
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TEAC	Trolox equivalent antioxidant capacity
WHO	World Health Organization

Introduction

A Brief History of Fluoride

Fluoride (F) is the 13th most abundant element in the Earth's crust and is present in the soil, water, air, animals, and plants. This element is found in many vegetables, beverages, and seafood (Cury 1981); however, the amount of F ingested by humans is difficult to be determined due to differences in food culture and industrialization (Buscariolo et al. 2006; Ozsvath 2009). F derivatives are naturally released into the

environment through volcanic activity, coal combustion, mineral dissolution, and marine aerosol production; in addition, they are also released as residues from industrial production (steel, aluminum, glass, bricks, ceramics, fertilizers, and pesticides). Moreover, the fluorine is the most electronegative and reactive element on the periodic table; therefore, it is rarely found naturally in its elemental state and reacts with other elements to form organic and inorganic F compounds (Miranda et al. 2021; WHO 2002).

Several important advances in science result from accidental observation, in which the observer initially oversees the potential consequences of the finding (Ten Cate and Buzalaf 2019). The hydrofluoric acid is known since the seventeenth century as well as that fluorspar emits light when heated; however, the high stability of F compounds prevented the discovery of the elemental state of fluoride for a long time. Only in 1886, the French chemist Henri Moissan succeeded in the isolation of fluoride through electrolysis with platinum and iridium (Moissan 1886) and received the Nobel Prize in chemistry in 1906 (Tressaud 2006).

The American dentist Frederick McKay invited the researcher Dr. Greene Vardiman Black to investigate the staining of teeth in his practice in Colorado (Ten Cate and Buzalaf 2019). They accidentally observed that patients with these enamel stains had a lower prevalence of caries than (McKay and Black 1916) children from other regions of the United States. After analyzing climatic conditions and eating habits, the researchers noticed that the drinking water source was the only difference between the groups and hypothesized that some chemical element would explain the occurrence of these stains (McKay 1928). Nevertheless, it took 30 years to determine that F in drinking water reduces the prevalence of caries (Ten Cate and Buzalaf 2019).

Since the industrial revolution, F was to be added to several products (oil, glass, agrochemicals, medicines, toothpaste) and food (meat, fish, cereals, juices, wines, processed foods, and some teas). In the 1950s, the World Health Organization (WHO) classified F as the third most polluting gas in the atmosphere that also contributes to ozone layer depletion (WHO 2000).

In 1945, pilot projects were started in Grand Rapids, Michigan, United States (Arnold et al. 1953, 1956; Arnold 1957), Newburgh, New York State, United States (Ast et al. 1950, 1951; Schlesinger et al. 1950; Ast and Schlesinger 1956), and Brantford, Ontario, Canada (Brown et al. 1956; Brown 1962; Brown and Poplove 1965), in order to investigate the safety, feasibility, and effectiveness of artificial fluoridation of public water on caries prevention. Since the relatively low cost and comprehensiveness of this strategy were also proven, several water fluoridation programs were soon after started throughout the United States and Canada, and F has been investigated worldwide (Narvai 2000).

Several institutions such as the WHO recommend the addition of F compounds into the water supply, which commonly uses sodium fluosilicate (Na_2SiF_6), fluorosilicic acid (H_2SiF_6), sodium fluoride (NaF), or calcium fluoride (CaF_2). The optimal F concentration is determined by the average temperature of each region (Agnelli 2016).

The significant reduction of caries prevalence observed since the 1970s is attributed to the expansion of preventive measures in developed countries and subsequently in developing countries as observed in two Brazilian surveys in 1988 and 1996 (Cangussu and Costa 2001; Brasil 1988). The ingestion of F through toothpaste and public drinking water plays a key role in the prevention and control of caries in children and adults (Pizzo et al. 2007).

In the medical field, F significantly contributed to the formulation of new drugs to treat osteoporosis and major depression disorder, such as the selective serotonin reuptake inhibitor named fluoxetine (Dure-Smith et al. 1991; Perez-Caballero et al. 2014). The several applications of this element that increase the human exposure to F are increased by its diversified applications/products and environmental and anthropogenic sources (Miranda et al. 2021).

Sources of Fluoride Exposure

Humans can be exposed to several sources of F such as drinking water, food, contaminated air, and occupational and topical products (Buzalaf and Whitford 2011; O'mullane et al. 2016). The underground water reservoirs are the biggest environmental source of F, in which levels can vary from 0.5 to 48 ppm (Susheela 2003). Highly industrialized cities in India and China can present F levels up to 46 ppm that lead to environmental contamination (Cao and Li 1992; Sankhla and Kumar 2018). The water supply is the main source of F in regions with a high prevalence of fluorosis (Mohr et al. 2018). Nevertheless, F has been added into domestic water supply networks due to its effectiveness in caries control (CDC 1999).

F is daily ingested through the consumption of products such as milk, salt, sugar, and herbal teas (Buzalaf and Whitford 2011; Wei et al. 2019). Moreover, the excessive use of some F-containing topical products such as toothpaste, mouth rinses, foams, and varnishes increases the F bioavailability and potentially causes detrimental effects in several human organs and systems (WHO 2002; Khan et al. 2018). F-induced bone pathologies are mostly investigated due to its high affinity to mineralized tissues; however, some studies have also shown that F can damage nonskeletal tissues such as the nervous system, heart, liver, kidneys, reproductive system, and thyroid (Bouaziz et al. 2006; Dec et al. 2017; Kuang et al. 2016; Iano et al. 2014; Wei et al. 2019) (Fig. 1).

Fluoride and Diseases

Fluorosis is a clinical condition resulting from chronic and dose-dependent exposure to F, which binds to Ca^{+} of mineralized tissues and modifies the chemical structure of hydroxyapatite ($Ca_{10}(PO_4)^6(OH)_2$) into fluorapatite (Ca_5 (PO_4) $_3F$) (Peckham and Awofeso 2014). Due to changes in crystal size and conformation, fluorapatite is more resistant to acid dissolution, which attenuates mineral loss and maintains balance in the demineralization of dental enamel (Tenuta and Cury 2010; Cury et al. 2019).

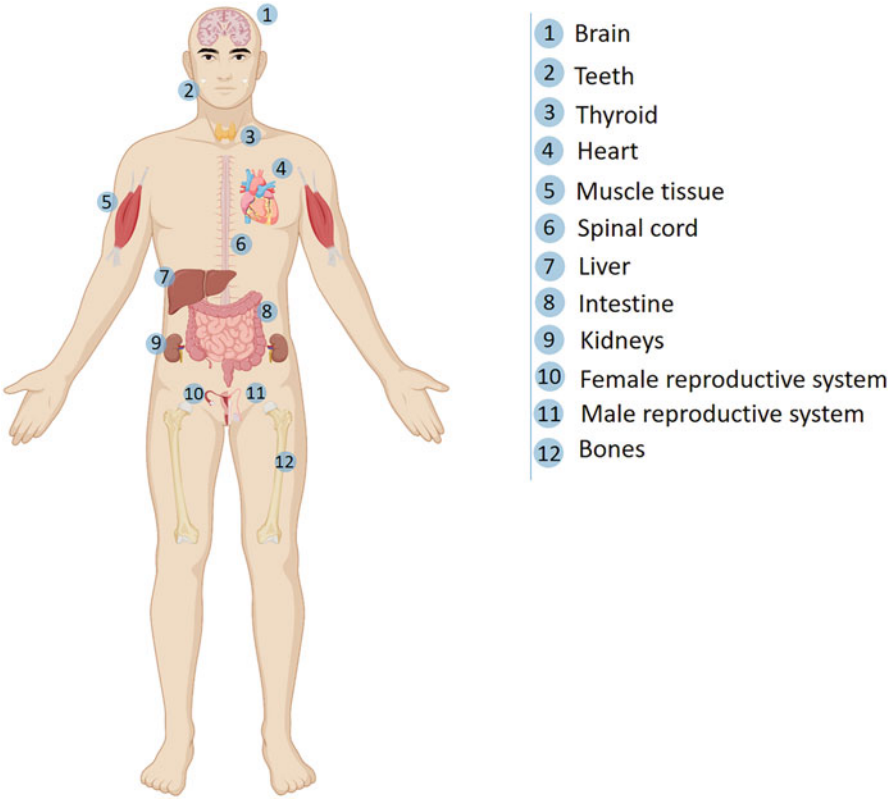

Fig. 1 Fluoride in the organism. Illustrative figure of the main organs and tissues affected by excessive exposure of fluoride

Dental fluorosis is a time- and dose-dependent pathology, in which a great amount of enamel matrix proteins are retained during the initial phase of mineralization and impair the ameloblasts to properly constitute hydroxyapatite crystals (Fujibayashi et al. 2011). Fluorotic enamel that is hypomineralized presents color and/or form changes (porous) and can be categorized into mild, moderate, or severe (Dean's Index) (Dean 1934; Cury et al. 2019).

Bone tissue also develops fluorosis, particularly during growth and remodeling due to the high absorption of F (Denbesten 1999). Scientific evidence suggests 1 mg F/L as the optimal dose of F ingestion (WHO 2017; Fawell et al. 2006); however, it can vary from 0.05 to 1.2 mg F/L depending on local temperature (Sampaio et al. 2010). Although WHO guidelines indicate that 0.05–1 mg F/L is safe for drinking water (WHO 2017), some individual's particularities such metabolism, tooth surface characteristics, and susceptibility to fluorosis need to be taken into account (Buzalaf 2018). Furthermore, there is no evidence that the therapeutic use of F causes any harm to human health (Cury et al. 2019).

In this context, skeletal fluorosis is a chronic metabolic disease caused by prolonged or excessive inhalation or ingestion of F that becomes toxic to the bones (Mohammadi et al. 2017; Watanabe et al. 2000). Since the systemic increase of F levels in the body is a long-term process, skeletal fluorosis has a higher incidence in elderly people as well as in workers of the steel industry that process CaF_2 (Krishnamachari 1986; Susheela et al. 2013).

Chronic exposure to F increases bone density during subperiosteal bone tissue formation. The decrease of osteoclast number, cortical bone thickening, a decrease of medullary elements, and irregular arrangement of chondrocytes cause structural and functional bone changes such as osteosclerosis, osteomalacia, osteoporosis, exostosis formation, and secondary hyperparathyroidism (Dhar et al. 2009; Medeiros et al. 2015). The vertebral column is the most commonly affected region, in which the ligaments crystallize in severe and chronic cases and lead to disabling deformities and neurological complications that are mainly mechanical (Reddy 2009).

F can also cause several degrees of extensive damage to non-mineralized tissues such as the common degenerative changes in parenchymal cells through inducing apoptosis to cells of different organs and tissues including lungs, kidneys, liver, brain, pancreas, thymus, endometrium, and bone marrow (Agalakova and Gusev 2012). Like other halogen elements, F can cross the blood-brain barrier and increase the production of free radical production in the central nervous system, which triggers the activation of several metabolic pathways related to Alzheimer's disease, Purkinje cell damage, and impairment of antioxidant defense systems (Valdez-Jiménez et al. 2011). Several studies suggest that 50 mg F/kg, which represents the exposure dose in areas with endemic fluorosis, damages several body systems at different life stages. It has been shown that pre- and postnatal exposure to F through placenta or breastfeeding modulates the oxidative balance and proteome homeostasis of the central nervous system particularly in the hippocampus of offspring (Ferreira et al. 2021). During adulthood, F can induce oxidative damage in the cerebellum that modulates some locomotor parameters (Lopes et al. 2020) as well as changes the levels of trolox equivalent antioxidant capacity (TEAC), thiobarbituric acid reactive substances (TBARS), concentrations of nitric oxide (NO), superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) in the peripheral blood circulation (Miranda et al. 2018).

Furthermore, an epidemiological study in an area with endemic fluorosis showed that excessive F intake is associated with the incidence of hypertension and carotid atherosclerosis in adults; in addition, the severity of the lesions may be related to increased plasma levels of endothelin-1 (Sun et al. 2013). Excessive F exposure can significantly increase hepatocytes apoptosis, promote the relative expression of caspase-3 and caspase-9 proteins, damage DNA, and disrupt lipid metabolism, which is the cause of liver oxidative stress response (Cao et al. 2013; Wei et al. 2018).

Renal histopathological studies have shown that F causes several degrees of damages in the architecture of tubular epithelium, endothelial cells, and mesangial cells of renal glomerulus. Excessive F intake alters the parameters of renal function.

Both oxidative stress and NF-κB signaling pathway play an important role in kidney damage, which eventually leads to inflammatory responses (Quadri et al. 2018; Luo et al. 2017). F reduces the viability of kidney epithelial cells and jeopardizes the function of epithelial sodium channels that are essential for body balance (Santesso et al. 2021). Excessive F exposure can also affect male sperm production and the function of the reproductive endocrine system, which decreases male reproductive capacity. The oxidative stress-mediated JNK and ERK signaling pathways induce the apoptosis of spermatogenic cells (Tian et al. 2018; Wei et al. 2018).

Therefore, several analyses have been developed to early diagnose alterations induced by toxic agents and consequently improve the individual's prognosis. Technological advances lead to more sensitive, robust, and noninvasive methods to detect biomarkers in body fluids and tissues and predict certain pathologies (Strimbu and Tavel 2010).

Biomarkers: A New Era for Diagnosis?

Biomarkers are biological indicators that indicate or even predict body alterations associated with pathologies (Buzalaf et al. 2018; Aronson and Ferner 2017; Grandjean 1995). Therefore, preclinical analyses of biofluids (blood, plasma, urine, feces, and saliva) or other tissues such as nails and hair can quickly detect biochemical alterations (Fig. 2). Biomarkers have shown cellular, molecular, and tissue aspects as potential indicators of several diseases and can be valuable for preventive medicine (Swenberg et al. 2008).

The investigation of biomarkers is essential to map and monitor areas with endemic fluorosis or even detect deficiency of F intake (Selwitz 1994). Therefore, all biofluids figure as a potential tool to early detect diseases and exposure to toxic agents or even aid to develop novel diagnosis methods (WHO 2000). Biomarkers can aid the early diagnosis or even predict pathologies regardless of their origin (Basu 2015).

Several well-established techniques can be used to detect or map biomarkers, such as real-time quantitative polymerase chain reaction (RT-qPCR), western blotting, and two-dimensional electrophoresis (Schrattenholz et al. 2012; Joseph 2017); in addition, omics analyses such as genomics, transcriptomics, proteomics, and metabolomics can be used to detect alterations at different molecular levels (genome, gene profile, protein, and metabolite expression, respectively). Although omics analyses are considerably accurate, their high cost still figures as a negative factor as well as their predictive character that requires clinical investigations.

Biomarkers and Fluoride: What's in the Literature?

Biomarkers figure as an excellent strategy to detect and monitor biological damage in individuals with specific pathologies or exposed to toxicants (Alves et al. 2014). Ekstrand et al. (1977) were one of the pioneer studies to use the now known biomarkers to detect F levels. Among the biomarkers investigated by several studies

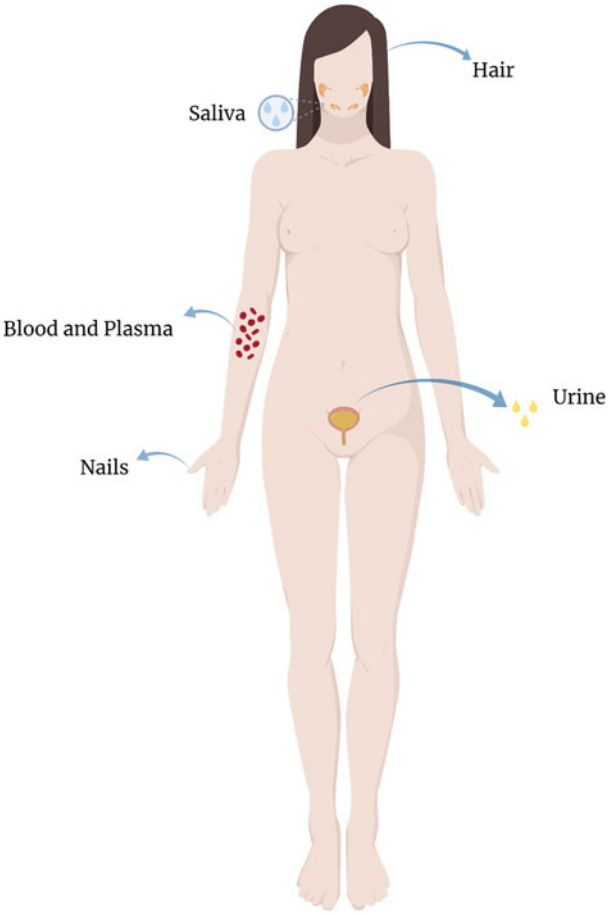

Fig. 2 Fluoride and biomonitoring. Illustrative figure of the main biological matrices, hair, and nails used to measure fluoride exposure levels in humans

for years, blood plasma and urine have shown the greatest sensitivity for quantifying F exposure. The association between F intake and plasma concentration is well elucidated in the literature (Carwile et al. 2020; Buzalaf and Whitford 2011; Ekstrand 1978); however, information such as the blood collection site, age, acid-base balance, altitude, hematocrit, and individual's genetic history need to be taken into account to reduce possible biases (Rugg-gunn et al. 2011; Whitford et al. 1999).

Urine is commonly used to measure F excretion and estimate daily exposure to F. This biofluid is considered a biomarker since a considerable part of the absorbed F is excreted in urine within 24 h, which variates in proportion among children and adults (Whitford et al. 1999; O'mullane et al. 2016). The use of saliva as a biomarker has been considered mainly due to its influence on the F concentration in dental plaque and caries control (Rugg-Gunn et al. 2011). Research on F level measurement

through saliva should consider the use of fluoridated dental products since increased F concentration in saliva has been associated with the use of toothpaste (Bruun and Thylstrup 1984; Pessan et al. 2008). In addition, the potential relation between F plasma concentration and parotid or submandibular ductal saliva has been reported (Oliveby et al. 1989; Whitford et al. 1999).

When considering F excretion, in particular, a proportional relationship between ingested F dose and F levels in the blood, saliva, and urine has been reported, which indicates saliva and urine can also be used as biomarkers of F exposure (Pessan and Buzalaf 2011).

Currently, scientific evidence shows that other tissues such as nails and hair can be useful as a noninvasive matrix to detect or measure levels of exposure to F or its compounds. Moreover, bioaccumulation of F in these tissues allows the identification of long-term exposures since their growth is supplied by the blood; thus, the average F plasma levels derived from chronic ingestion can better indicate short- and long-term F exposure from months to years (Elekdag-Turk et al. 2019). Toenails are particularly considered the main epidemiological matrix for monitoring chronic and subchronic F exposure in regions with fluoridated water (Lavalle-Carrasco 2021). A significant correlation between F consumption and its concentration in nails was observed in 2–6-year-old preschool children living in a city with fluoridated water, while this was not observed in a negative control population living in a non-fluoridated city (Levy et al. 2004).

Hair samples have also been widely used for biomonitoring exposure to F and its compounds in areas where fluoridated water is the main source of human exposure (Lavalle-Carrasco 2021). Analyses of hair samples from adults living in areas with endemic fluorosis showed higher levels of F than those hair samples collected from inhabitants of non-endemic areas (Joshi and Ajithkrishnan 2018; Rugg-Gunn et al. 2011). In addition, a positive correlation between F levels in drinking water and F concentration in hair samples was observed in children from 7 to 15 years of age (Antonijevic et al. 2016). Long-term biomonitoring of child and youth populations is essential to reduce the deleterious effects of F exposure, particularly in areas with several sources of F exposure (Levy and Zarei-M 1991; Till et al. 2020).

Different from breast milk, F concentration in plasma proportionally increased with F intake and showed the greatest equivalence after 30 min; thus, it has been suggested that plasma is a biomarker of great sensitivity to F (Ekstrand et al. 1981). F has a plasmatic half-life of 30 min, and its blood concentration returns to baseline after 3–6 h after F intake. In addition, F concentrations in saliva correspond to plasmatic concentrations. Therefore, urine has been suggested as the more appropriate biomarker of F acute exposure since it is the main form of F excretion (Pessan and Buzalaf 2011).

A systematic review has indicated that biofluids such as plasma, urine, and saliva can be used to investigate immediate and short-term exposure to fluoridated compounds; however, urine is better for predicting total daily fluoride intake (Lavalle-Carrasco 2021). The use of F-containing topical products such as toothpaste and mouth rinses may bias the F level in saliva, while the use of plasma as a biomarker represents an invasive sample collection and is more difficult to obtain in epidemiological surveys in areas with endemic fluorosis. Therefore, the choice of the most

appropriate biomarker depends on the respective study aim (Pessan and Buzalaf 2011; Rugg-Gunn et al. 2011).

Recent studies have used the specific electrode method with hexamethyldisiloxane (HMDS)-facilitated diffusion to measure the F concentration in plasma or other aqueous matrices. HMDS separates F from the sample, eliminates interferences, and simultaneously concentrates it; thus, F detection threshold is increased by sensitive ion-specific electrodes (Taves 1968; Whitford 1996). Since the detection threshold of the electrode is similar or even higher than plasma concentrations (approximately 0.019 mg/L), this technique has high sensitivity and low bias potential (Whitford et al. 1999).

Conclusion

Both acute and chronic long-term F exposure can lead to several detrimental effects. In addition to soft tissue pathologies, chronic exposure to high F doses may result in skeletal and dental fluorosis in the bones and teeth, respectively. The determination of the most suitable biomarker for F biomonitoring needs to take into account the exposure period and population characteristics.

Mini-Dictionary of Terms

- Dental fluorosis: a disease that affects the dental elements when there is exposure to high levels of fluoride during the period of formation of the dental elements.
- Skeletal fluorosis: a disease that affects the bone tissue; its signs and symptoms can be similar to other bone pathologies.

Key Facts

Fluoride is used for various purposes, such as in mining, pharmaceutical industry, manufacturing of toothpastes, and fluoridation of domestic water supply.

Throughout history, the safety and effectiveness against tooth decay in humans conferred to fluoride have been tested and recommended by regulatory bodies around the world.

Dental and skeletal fluorosis are the manifestations most commonly associated with prolonged exposure to fluoride; however, they have different clinical characteristics.

Summary Points

- High concentrations can break homeostasis of various tissues and systems in humans.

- Clinical manifestations may vary between individuals due to genetic factors and renal clearance.
- Several biofluids are useful in biomonitoring fluoride exposure.

Cross-References

► Biomarkers of Neurotoxicity
► Oxidative Stress Biomarkers and Their Applications to Detect Excessive Fluorine

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New Approaches to Evaluate the Cytotoxic Potential of Leishmanicidal Drugs Using Human Peripheral Blood

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Abstract

Parasite resistance and few therapeutic options are challenges that need to be overcome for effective treatment of leishmaniasis. In the drug development pipeline, new alternatives of leishmanicidal agents must be evaluated for their toxic impact on innate and acquired immunity cells, considering that the pharmacological modulation of the immune response is essential for the success of the leishmanicidal activity. Among the in vitro methods for immunotoxicity measurement, there are the conventional assays using human and animal cells. Animal data provide little evidence that there will be no adverse reactions in humans. Human peripheral blood leucocytes have been used as a more promising model to predict toxicity of new leishmanicidal agents. This chapter discusses approaches using conventional models to test the toxicity of leishmanicidal agents, the cell types used, and the importance/applicability of new functional assays with human peripheral blood cells employing multiparametric analysis by flow cytometry for preclinical assays in drug discovery.

Keywords

Leishmaniasis · Neglected disease · Drug discovery · Leishmanicides · Whole blood · Immunotoxicity · Chemoimmunotherapeutic · Preclinical assays · 3R · Predictive assay

List of Abbreviations

7AAD	7-Aminoactinomycin D
ADME	Absorption-distribution-metabolism-excretion
AmB	Amphotericin B
ATP	Adenosine triphosphate reduction
AUC	Area under the curve
Calcein-AM	Calcein acetoxymethyl
CBA	Cytometric bead array
CD	Cluster of differentiation
CCL2	C-C Motif Chemokine Ligand 2
CCL4	C-C Motif Chemokine Ligand 4
CD11b	Cluster of differentiation 11b
CD16	Cluster of differentiation 16
CD66b	Cluster of differentiation 66b
CD14	Cluster of differentiation 14
CD3	Cluster of differentiation 3
CL	Cutaneous leishmaniasis
CXCL8	C-X-C Motif Chemokine Ligand 8

Da	Dalton
DAPI	6-Diamidino-2-phenylindole
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
DNDi	Drugs for Neglected Diseases initiative
ECVAM	European Centre for the Validation of Alternative Methods
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated single cell sorting
FDA	Food and Drug Administration Agency
FDA	US Food and Drug Administration
FITC	Fluorescein isothiocyanate
GF-AFC	Glycylphenylalanyl-aminofluorocoumarin
GHIT	Japanese Global Health Innovative Technology
HPL	Human peripheral leukocytes
IC_{50}	Half-maximal inhibitory concentration
IFN-γ	Interferon gamma
IL-1β	Interleukin 1 beta
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-15	Interleukin 15
IL-18	Interleukin 18
LDH	Lactate dehydrogenase
LEAD	A chemical compound or natural product that has biological activity against a drug target
M1	Macrophages phenotype 1
M2	Macrophages phenotype 2
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
MW	Molecular weight
NETs	Neutrophil extracellular traps
NO	Nitric oxide
PD	Pharmacodynamics
PE	Phycoerythrin
PGE2	Prostaglandin E2
PK	Pharmacokinetics
R&D	Research and development
rHuGM-CSF	Human recombinant granulocyte-macrophage colony-stimulating factor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SbIII	Trivalent antimony
SbV	Pentavalent antimony
SDH	Succinate dehydrogenase

TGF-β	Transforming growth factor beta
Th1	Type 1 T helper cells
Th2	Type 2 T helper cells
TL	Tegumentary leishmaniasis
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
VL	Visceral leishmaniasis

Leishmaniasis: Neglected Tropical Diseases Are Still Spreading Around the World

Leishmaniasis are infectious diseases of zoonotic or anthroponotic nature caused by parasitic protozoa of the order Kinetoplastida, family Trypanosomatidae, and genus *Leishmania*. These diseases are caused by over 21 different *Leishmania* species, which are transmitted to humans by the bite of female phlebotomine sandflies. Currently, the parasites are classified into two subgenres – *Leishmania* and *Viannia* – and this classification considers both phylogenetic molecular markers and developmental characteristics of the parasites in the insect vector (Boité et al. 2012).

Leishmaniasis can comprise two main clinical forms: tegumentary leishmaniasis (TL) characterized by cutaneous and/or mucosal lesions and visceral leishmaniasis (VL), a systemic form that is lethal if not treated. The occurrence of different clinical forms is due to different factors, such as the composition of the vector's saliva; the amount of *Leishmania* inoculated during the blood meal; age and the nutritional and immunological status of the host; and mainly the tropism and virulence of the *Leishmania* species (Lipoldová and Demant 2006).

Leishmania parasites have two morphologically and biochemically distinct forms: amastigote and promastigote. The amastigote forms are rounded, without an apparent flagellum, and infect and multiply in cells of the mononuclear phagocytic system of the vertebrate host. The promastigote forms are elongated, flagellated, and mobile and live in the lumen of the digestive tract of the sand fly (Balaña-Fouce et al. 1998). The parasites of the genus *Leishmania* have a digenetic or heteroxenic life cycle, whose forms of development alternate between invertebrate and mammalian hosts. The invertebrate hosts (insect vectors) are females of the order Diptera, family Psychodidae, subfamily Phlebotominae, and genus *Lutzomyia* (New World) and *Phlebotomus* (Old World) (Bates 1994). Cell cycle of leishmaniasis is described in Fig. 1.

Leishmaniasis are classified by the World Health Organization (WHO) as neglected tropical diseases, being distributed in 98 countries in Africa, Asia, the Americas, Europe, and Oceania. More than 58,000 cases of VL and 220,000 cases of TL are reported annually worldwide. In addition to a large number of annual cases, leishmaniasis causes high mortality: around 20,000 to 30,000 deaths per year (Alvar et al. 2012; Kevric et al. 2015; WHO, 2021). Due to the spread of leishmaniasis on several continents and the occurrence of severe clinical forms, leishmaniasis was considered one of the diseases of priority attention by the WHO (Alvar et al. 2012).

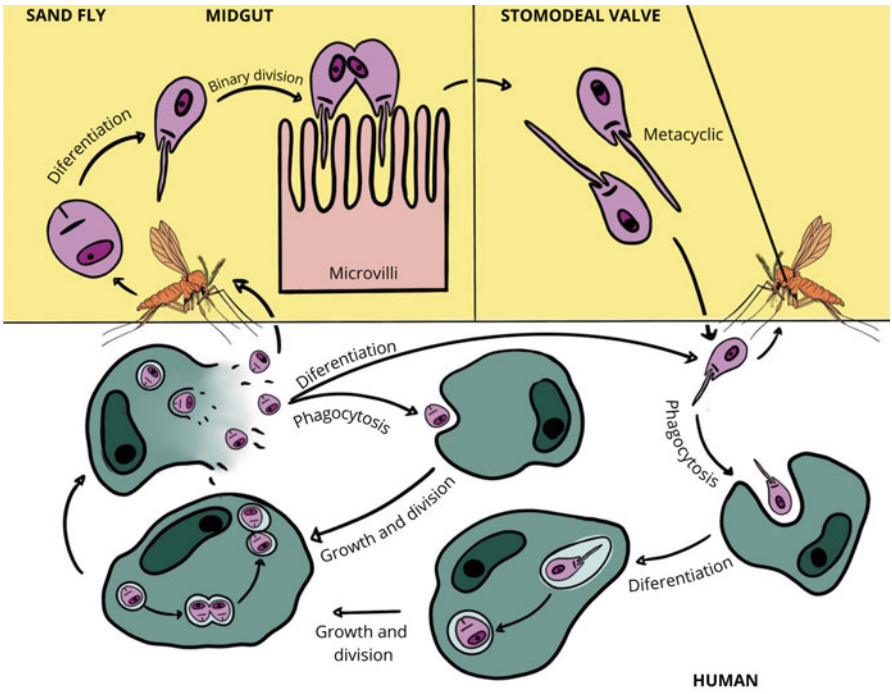

Fig. 1 The cell cycle is central to understanding of *Leishmania* biology.

Leishmania have two main life cycle. In the sand fly, the promastigote forms live in the lumen of the digestive tract (midgut and stomodeal valve), where they multiply by binary division and differentiate into the infecting metacyclic form. Already and in the mammalian host (e.g., human), after the bite of infected female phlebotomine sand flies, the promastigotes are phagocytized by monocytes/macrophages and transform in intracellular amastigotes forms, which multiply by binary division and can infect other monocytes/macrophages

Leishmaniasis Treatment

Chemotherapy is the main form of leishmaniasis control since there is no human vaccine available. Currently, a small number of drugs are available for the treatment of leishmaniasis: pentavalent antimonials, amphotericin B, miltefosine, paromomycin, and pentamidine (Muraca et al. 2020).

Pentavalent antimony-containing compounds (Sb^V) (e.g., sodium stibogluconate, Pentostam®, and meglumine antimoniate, Glucantime®) have been used as the first-line treatment against all forms of leishmaniasis in many countries (Croft et al. 2006). The mode of action of Sb^V is still not completely understood. It is generally agreed that Sb^V is a prodrug that becomes active when it is reduced to the trivalent form (Sb^{III}) (Goodwin 1995; Ponte-Sucre et al. 2017). Some studies suggest that the activation of antimonials can occur in macrophages (Brochu et al. 2003), while other studies indicate that the reduction can happen inside the parasites (Frézard et al. 2014).

Treatment failure with pentavalent antimony (Sb^V) has been reported in different countries, especially in the state of Bihar in India, where more than 60% of VL patients do not respond to treatment with antimonials (Thakur et al. 2001).

Amphotericin B is the most potent leishmanicidal drug currently available, and it can be used in the treatment of *Leishmania*sis because it has an affinity for ergosterol, the main sterol present in these pathogens. Mammalian cells, on the other hand, use cholesterol and are, therefore, less sensitive to the drug (Ramos et al. 1996). The action of amphotericin B on the parasite membrane makes it more permeable to ions, thus inducing cell death (Ramos et al. 1996). The mechanisms of resistance to amphotericin B include the change in membrane fluidity and increased expression of genes associated with the thiol pathway (Purkait et al. 2012).

Miltefosine (hexadecylphosphocholine) is a phosphatidylcholine analog initially developed as an antineoplastic drug shown to be very effective for VL treatment in India (Sundar et al. 2002). This drug is the only one administered orally against leishmaniasis. Miltefosine has been linked to lipid metabolism disruption, mitochondrial malfunction, abnormalities in cell proliferation, initiation of apoptosis-like cell death, and immunomodulatory effects (Paris et al. 2004; Loiseau and Bories 2006; Marinho et al. 2011; Dorlo et al. 2012).

Therapeutic failure of leishmaniasis is a growing concern and may be related to the occurrence of drug-resistant parasites but also to other factors associated with the host, such as the immune system and genetic factors of the patient; to the drugs, such as pharmacodynamics/pharmacokinetics (PD/PK); and to others (Ponte-Sucre et al. 2017). The occurrence of drug-resistant parasites is mainly related to the great diversity of parasites in this genus and the high plasticity of the genome, such that resistance can occur by altering different metabolic pathways, demonstrating that the phenomenon of drug resistance is complex and multifactorial (Santi and Murta 2022). Leishmaniasis chemotherapy presents several issues, such as high drug toxicity, long treatment protocols, the limited choice of drugs, and the occurrence of cases of therapeutic failure (Singh et al. 2012). Thus, the search for new drugs for effective treatment against leishmaniasis is an urgent need.

In this chapter, the importance of new leishmanicidal drugs and the role of the immune system for therapeutic success, the pipeline of drug discovery, and the challenges of developing preclinical predictive assays to search for new compounds with anti-*Leishmania* activity are described.

Leishmaniasis: The Immune Response for Parasite Control and New Drug Targets

Leishmania is an obligate intracellular parasite that rapidly infects phagocytic cells. The immune response to *Leishmania* infection is primarily initiated by innate immune cells which orchestrate the generation of protective innate and adaptive immunity against parasites. In this context, chemokines and cytokines play an important role in determining cellular composition at inflammatory sites during the

infection, and the balance and dynamic changes in their levels may control or predict the disease outcome (de Araújo et al. 2021).

As the host immune response is a major determinant for the outcome of *Leishmania* spp. infection, a better understanding of the immunological spectrum is necessary to transpose into effective actions associated with disease outcome, such as therapeutic schemes. In this sense, numerous evidence point out the importance of the innate immunity as a target for new studies for the treatment of leishmaniasis.

Innate cells such as neutrophils and mononuclear phagocytes (monocytes, macrophages, and dendritic cells – DCs) create both a hostile and an accepting environment for the parasite, consequently directing the adaptive immune response (Rossi and Fasel 2018).

Neutrophils are the first cells recruited to the site of *Leishmania* spp. infection and are responsible for the formation of neutrophil extracellular traps (NETs); phagocytosis; production of reactive oxygen species (ROS); nitric oxide (NO); several cytokines such as IL-12, IFN-γ, TNF, IL-10, and TGF-β; granule proteins; and eicosanoids, all elements that participate in the modulation of adaptive immunity (Hurrell et al. 2016). Neutrophils, in addition to their independent role in orchestrating the early cytokine/chemokine responses, also interact with other immune cell types including DCs and macrophages. In this sense, in human macrophage/neutrophils *L. infantum*-infected co-cultures, was observed an increase in TGF-β and PGE_2 (Teixeira et al. 2018). Moreover, parasitized neutrophils may facilitate *Leishmania* uptake by DCs leading to DC deactivation, impairing the T cell *Leishmania*-specific function (Kupani et al. 2021). Thus, owing to their capacity of antigen presentation and of stimulation of T cell activation, similarly to mononuclear phagocytes, neutrophils may be relevant to leishmaniasis treatment as in vaccinal schemes. In this context, recent vaccine studies have shown that neutrophils may acquire nonspecific memory by epigenetic and metabolic changes, characterized as "trained immunity" (Netea et al. 2016).

The recruitment of monocyte subpopulations (classical and nonclassical, as pro-inflammatory monocytes) is dependent on the soluble immune mediators' microenvironment, such as provided by chemokines and cytokines, after *Leishmania* spp. infection. Monocytes can recognize the parasites via Toll-like receptors (TLRs) and produce pro- and anti-inflammatory cytokines that can present contradictory roles. *L. donovani* infection inhibits oxidative burst and antigen presentation and induces IL-10 production associated with modulation of TLR2/TLR4 signaling (Roy et al. 2015). Furthermore, studies from our group (Peruhype-Magalhães et al. 2005) and India (Roy et al. 2018) evaluating visceral leishmaniasis (VL) patients demonstrated an increase of $CD14^+$ monocytes and elevated expression of IL-10, an important cytokine implicated in macrophage polarization and disease outcome. Other studies also reported an anti-inflammatory monocyte response characterized by reduced expression of TLR2/TLR4, chemokine receptors, and adhesion molecules, as well as impaired phagocytosis and oxidative burst, in VL patients (Singh et al. 2018). Recently, several studies have focused on explicating the role of monocyte subsets in *Leishmania* spp. infection.

Pro-inflammatory monocytes contribute to the control of disease in cutaneous leishmaniasis (Costa et al. 2016). These cells are an important source of NO during

skin infection and produce IL-18 and IL-15, which can play a critical role in the differentiation of T cells to a memory phenotype. Thus, due to their roles in parasite clearance at the site of infection, pro-inflammatory monocytes could enhance the efficacy of anti-*Leishmania* treatment. On the other hand, pro-inflammatory monocytes play a damaging role in VL (Terrazas et al. 2017). In this context, regulating monocyte migration with immunomodulatory agents, for example, could be a potent parasite control strategy (Rosas et al. 2006). Indeed, therapeutic schemes that reduced pro-inflammatory monocyte influx, such as treatment with Ibrutinib, have been shown to decrease susceptibility to *L. donovani* infection (Varikuti et al. 2019).

It is important also to highlight the role of macrophages (M1/M2 phenotype), relevant host cells for the parasites, following phagocytosis of apoptotic neutrophils (van Zandbergen et al. 2004). Infection of macrophages by *Leishmania* spp. is accompanied by metabolic changes that improve the parasite intracellular survival and, subsequently, contribute to the differentiation of anti-inflammatory macrophages (M2), via IL-13 and IL-4, using a mechanism independent of TLR activation (Van den Bossche et al. 2017). Nevertheless, pro-inflammatory (M1) macrophages upregulate IL-12 and TNF cytokines, as well as ROS and NO production, crucial elements to parasitism control. In this inflammatory microenvironment, IL-12 can promote the differentiation of Th1 lymphocytes. IFN-γ expression by Th1 lymphocytes induces production of IL-12 and NO which stimulates macrophage leishmanicidal activities (Rostami and Khamesipour 2021). Considering the role of macrophages in inducing an immune response against the parasite and due to their role as professional antigen-presenting cells directing the immune response to Th1 or Th2 patterns, these cells have been evaluated as candidates for therapeutic approaches.

In this context, it is important to mention alternative approaches for leishmaniasis treatment able to induce activation of the immune system, especially of infected target cells. Amphotericin-B (AmB) is a potent pro-inflammatory stimulant of innate immune cells. The treatment exhibits efficient results in leishmaniasis since AmB has two effects on the parasite: binding to the ergosterol at its membrane, causing pore formation in *Leishmania* spp., and stimulating oxidative stress mechanisms in macrophages and, consequently, accumulation of intracellular ROS (Mesa-Arango et al. 2012). In addition, AmB induces the production of chemokines (CXCL8, CCL2, and CCL4) and pro-inflammatory cytokines (TNF, IL-6, and IL-1β) by neutrophils and mononuclear phagocytes (Ben-Ami et al. 2008). A summary of the immunological aspects associated with human leishmaniasis is presented in Fig. 2.

To date, there is no effective vaccine and the traditional treatments for leishmaniasis are toxic and of long administration duration and frequently present many adverse side effects besides drug resistance (Akbari et al. 2021). In this sense, combined therapy may be considered as an important option for *Leishmania* treatment. Immunotherapy that supports activation of the immune response, associated with chemotherapy, may resolve the complicated cases of the disease mainly in the instance of treatment refractoriness. On HIV/VL coinfection, for example, the combined therapy using AmB and rHuGM-CSF showed to be effective, decreasing

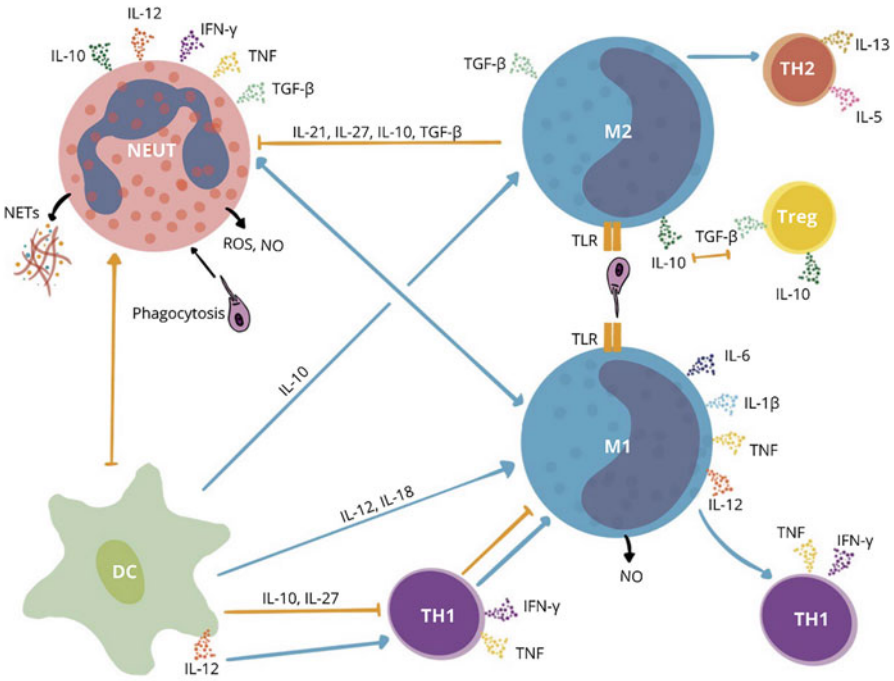

Fig. 2 Overview of the immunological aspects associated with human *leishmaniasis*.

Neutrophils are the first cells recruited to the site of *Leishmania* spp. infection and are responsible for the formation of neutrophil extracellular traps (NETs), phagocytosis, and production of reactive oxygen species (ROS), nitric oxide (NO), and several cytokines such as IL-12, IFN-γ, TNF, IL-10, and TGF-β. Moreover, parasitized neutrophils may facilitate parasite uptake by dendritic cells (DCs) leading to deactivation of this cell. The recruitment of monocyte and macrophage differentiation (M1/M2 phenotype) is dependent of the immune mediators' microenvironment. The macrophages can recognize the parasites via Toll-like receptors (TLRs) and the infection is accompanied by metabolic changes that improve the parasite intracellular survival and, subsequently, contribute to the differentiation of anti-inflammatory macrophages (M2), via IL-13 and IL-4. Nevertheless, pro-inflammatory (M1) macrophages upregulate IL-12 and TNF cytokines that promote the differentiation of Th1 lymphocytes. TNF and IFN-γ expression by Th1 lymphocytes induces production of NO which stimulates macrophage leishmanicidal activities

the spleen size, helping in the disappearance of symptoms and clinical cure (Mastroianni 2004), and making the use of immunomodulators the most promising strategy in therapeutic vaccines for leishmaniasis.

Chronic infections with *Leishmania* can induce prominent changes in host metabolomic pathways, which can influence immune cell proliferation, differentiation, and its effector functions. Current advances in metabolomic profiling during leishmaniasis, especially for innate immune cells, may reveal novel immune regulation networks that can be exploited for vaccine development (Volpedo et al. 2021). Instead of treatments based on conventional chemotherapy, certain alternative strategies that aim to reduce the parasitic burden and promote recovery of leishmaniasis

patients are currently in study and have been focused on the induction of effective immune responses to rapidly control the disease.

New Drugs Are Needed!

Parasite resistance and few therapeutic options currently available are challenges that need to be overcome for the effective treatment of leishmaniasis. As a disease that affects poor countries and consequently does not generate profit, the pharmaceutical industry neglects it by not investing R&D efforts to make new drugs available on the market (Sunyoto et al. 2018). Among the available treatments, pentavalent antimonials, amphotericin B, and miltefosine are classified within the approach known as chemoimmunotherapeutic. That is, they stimulate the immune response in addition to their direct action on the parasite, which enhances the leishmanicidal effect (Aruleba et al. 2020). Amphotericin B and antimonials are parenteral and require hospitalization, and the treatment is long and expensive (Sunyoto et al. 2018). In addition, they can cause unwanted effects such as cardiotoxicity, gastrointestinal problems, and liver, kidney, and pancreatic toxicity (Ferreira and Andricopulo 2018). The growing resistance of the parasite to drugs continues to be a great challenge, and programs to discover new leishmanicidal drugs are underway.

In the drug discovery pipeline, during screening and preclinical phases, new chemical entities are tested in cellular models, among others, which allow the identification of pharmacological efficacy and toxicity. Mononuclear phagocytic cells of the immune system such as monocytes and neutrophils are the cells where *Leishmania* parasite proliferates, before spreading to other organs. For that reason, in vitro and in vivo assays in this context should mimic with accuracy and predictability the natural infection to evaluate leishmanicidal and cytotoxic activities.

Challenges in the Drug Discovery for New Leishmanicidal Drugs

The anti-*Leishmania* drug discovery is still a high-cost and time-consuming approach, and there is no established pipeline to identify and optimize new candidates since it has several challenges and gaps (Balãna-Fouce et al. 1998). The drug discovery process encompasses several steps, from target identification to its use in the market.

Generally, the approach at discovery phase is to perform the screening (Fig. 3, second step) of a library of compounds against target-based assays, a validated molecular/biochemical target, or cell-based validated phenotypic assays. In this stage of the initial target-based approach, the experts have to deal with target identification and validation (Fig. 3, first step) (Balãna-Fouce et al. 1998; Alcântara et al. 2018). Compounds are selected considering their potency, based on their IC_{50} values on anti-*Leishmania* and cytotoxicity models, preferably on assays using mammalian cell lines. In this stage, testing the compounds against some strain panels is important to identify the promising compounds (Fig. 3, second step)

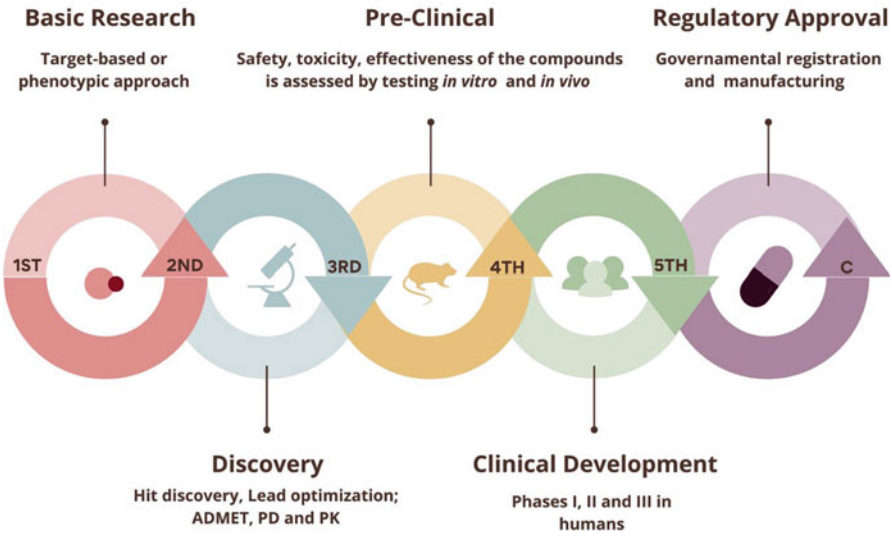

Fig. 3 Classical stages of drug discovery pipeline for *leishmanicidal* compounds.
The process includes several stages which occur sequentially: (first) basic research (target or phenotypic assays), (second) discovery of the drugs (hit discovery, lead optimization, and ADME, TOX, PD, and PK studies), (third) preclinical assays (safety, toxicity, and effectiveness of the compounds by in vitro and in vivo assays), (fourth) clinical development (phases I, II, and III in humans), (fifth) regulatory approval (governmental registration and manufacturing), and (C) commercialization

(Balãna-Fouce et al. 1998). After identification of active compounds, they go further to the development phase to check toxicity, efficacy, safety, ADMET (absorption, distribution, metabolism, excretion, and toxicity), and PK and PD properties in different preclinical in vitro and in vivo models (Fig. 3, third step). LEAD compounds and new chemical entities with a good profile of PK, PD, safety, and metabolism meet the criteria to be selected as drug(s) candidate(s) that will follow into the late state of preclinical and clinical development to finally reach the market. Regulatory approval of a new drug depends on the ADMET properties, which indicates that the compound is safe and effective to be commercialized.

The development phase of clinical trials (I, II, III) (Fig. 3, fourth step) involves human subjects to assess the efficacy, tolerability, and safety of the compounds, PK/PD parameters, and the impact of food intake on the absorption of the drug. The main goals are as follows: phase I: it is a small study with few numbers of individuals (dozens) to determine the pharmacokinetics, safe dose range, and common side effects in healthy human volunteers; phase II: it is a moderate-size study (hundred individuals) to determine efficacy and less common side effects; and phase III: these are large (thousand individuals) prospective studies to confirm safety and efficacy in patients (Van Norman, 2016).

The last steps of the pipeline include the manufacture of the medicine and registration that depends on each federal country's law (Fig. 3, fifth step). In general,

for registration, all information about the drug that covers clinical trials, manufacturing process, quality control, and assurance is required. The approval of a new medical drug in the United States by the FDA application can take an average of 12 years and costs above US$1.3 billion, and the overall probability of a drug passing all stages is approximately 11% (Van Norman, 2016).

The Drugs for Neglected Diseases initiative (DNDi), a nonprofit organization, and their consortium of R&D partners have sought short-course treatments for leishmaniasis with existing drugs, particularly for children, and new oral treatments for both forms of leishmaniasis, including combinations of new chemical entities that are safe and easier to manage at the primary healthcare level. The DNDi's portfolio has drugs in different stages of development, from optimizing the best active compound to drugs in the preclinical phase. Treatment regimens are being developed based on symptoms in relation to the different species of leishmania and the geographic peculiarities of these parasites. This consortium has contributed to accelerating the discovery of new chemical entities, the optimization of LEAD candidates, the translation process and their scale-up, and the drug's repositioning processes (DNDi, 2020).

A Quick Look at In Vitro Assays to Screen New Chemical Entities for Leishmania and Existent Preclinical Assays

In vitro screening assays can be performed on different stages of the *Leishmania* cycle (promastigotes, axenic amastigotes, or intracellular amastigotes). Advantages and limitations should be considered in these preliminary screening studies such as the ability of the drug to penetrate in the host cell and be active in acidic milieu, the high false-positive rates of the assay, the relative absence of correlation between assays using axenic and intracellular amastigote forms, the different susceptibilities of intracellular amastigotes to drugs considering incubation time and the different *Leishmania* species, the need of testing the drug against a panel of strains (and clinical isolates), and the virulence fluctuation over time of laboratory-adapted parasites (De Rycker et al. 2013; Morais-Teixeira et al. 2014; Alcântara et al. 2018; Baek et al. 2020).

The drug susceptibility is also dependent on host cell type since intracellular *Leishmania* can determine its velocity of replication. The most used host cells in in vitro amastigote assays are the primary cells such as bone marrow-derived macrophages (BMDM) and peritoneal macrophages or macrophages obtained from transformed murine strains such as J774A1, J774.G8, and RAW264.7 cell lines (Baek et al. 2020). Monocytic-derived cell lines are preferred for their reproducibility and ability to yield a homogenous population of cells for the screening assays. Transformed human leukemic lines such as THP-1, JE6.1, U937, and HL60 are stimulated with phorbol 12-myristate 13-acetate (PMA) to produce cells with macrophage-like properties (Kashif et al. 2019; Zulfigar et al. 2017). The human monocyte/macrophage cell line THP-1 is the most used model, not only for leishmanial studies but also for other diseases caused by intracellular pathogens such as

Mycobacterium tuberculosis, *Listeria monocytogenes*, *Legionella* (Pick et al. 2004), *Plasmodium falciparum*, and HIV-1 (Berger et al. 2017). Toxicity is likewise evaluated in other models including human cells as peripheral blood mononuclear cells, monocytes, and malignant HepG2 hepatocytes (Norcliffe et al. 2018). Thus, based on compound susceptibility differences due to host cell type and parasite strains, many studies have claimed validated and standard tests to evaluate anti-leishmanial drugs (Baek et al. 2020; Hefnawy et al. 2018; Hendrickx et al. 2019).

The active(s) compound(s) identified at the screening step are tested in animal models to characterize their pharmacokinetic and pharmacodynamic properties. In this stage, the promising compounds, which are named "LEAD compounds," display greater selectivity than 100-fold for the pathogens that cause diseases (e.g., visceral leishmaniasis), have good oral bioavailability and no acute toxicity at in vivo efficacy studies such as cardiac safety and in vitro safety parameters (genotoxicity and the mini-Ames test), and are devoid of carcinogenicity, mutagenicity, and teratogenicity (Katsuno et al. 2015). Another important parameter in this stage is the time-to-kill assay that assesses the cidal activity dynamics and identifies the minimal exposure time needed to completely eliminate viable intracellular amastigotes. This is also a parameter that supports the establishment of dose regimens (Maes et al. 2017).

For cutaneous leishmaniasis, there is no validated animal model since several species are causative agents implying different manifestations (Alcântara et al. 2018). For the drug discovery process, the in vivo efficacy in mice, lesion suppression model, and mouse efficacy for lesion cure model are assessed. The candidate drug should reduce at 95% the lesion in a mouse model with *Leishmania tropica* or in the golden-hamster model with *Leishmania brasiliensis* for entering in the preclinical assays (Caridha et al. 2019; Alcântara et al. 2018). The active compound in vivo is developed based on ADMET criteria, its toxicology properties, and drug-drug interactions (Ekins et al. 2019; Caridha et al. 2019; Alcântara et al. 2018).

In the preclinical steps for the drug development, wide doses of the drug are tested in in vitro and in vivo experiments. The potential targets for pharmaceutical action and the chemicals that modify the targets are identified, and the in silico profiling using computer models of the drug-target interactions can be assessed for optimizing undesirable side effects by inference of unwanted targets. Besides including the information about the involved biological system, the preclinical trials are the beginning of the development of pharmaceutical formulation and the sketch for the scaling up of the manufacturing process (Gail and Van Norman 2019).

One of the problems in the pipeline drug discovery is that several compounds which are mainly identified either through random screening or are repurposed, although they display potent in vitro antileishmanial effects, do not have efficacy in the in vivo system. *Leishmania* parasites reside primarily in the spleen and liver, and the drug should accumulate in these organs. If the drug orally administered is metabolized too fast or has less oral bioavailability, its effectiveness will decrease (Goyal et al. 2021). The preclinical studies support the clinical trials in humans. Although pharmacokinetic and pharmacodynamics studies are limited, they are now needed to assess safety, efficacy, and drug posology for humans (Alves et al. 2018).

Therefore, for drug tests, the most predictive models must simulate in vitro the intracellular form of the parasite in monocyte/macrophages and neutrophils, which represents the natural infection using cell models derived from humans and/or other species (Tavares et al. 2018). These cell types are evaluated about the infection by the parasites and the impact of the test compounds on their viability, once it is desirable that the chemicals kill the parasite, with no damage or minimal damage to the host cell.

There are still challenges regarding models that use cells derived from animals or tumor lines, which has encouraged the development of more predictive assays to search for new leishmanicidals.

Current Assays to Measure the Cytotoxicity of Chemical Entities

In the current protocols described in the literature to test chemical entities, cell functions such as enzymatic activity and membrane integrity are the parameters more used in cell-based models. The quantification of cytotoxicity in most of these assays is done by spectrophotometer, microscopy, or flow cytometry after labeling with colored, fluorescent, and luminescent dyes (Fig. 4, a and b).

The so-called vital exclusion dyes used in the permeability assays are lower cost and quick protocols to measure cell viability. They include colored dyes like trypan blue or fluorescent ones like propidium iodide, 7-aminoactinomycin D (7AAD), and 6-diamidino-2-phenylindole (DAPI). These dyes permit distinction between living cells from dead ones based on the selectivity of the cell membrane, i.e., they cannot cross the intact membranes of viable cells and are selectively excluded by them.

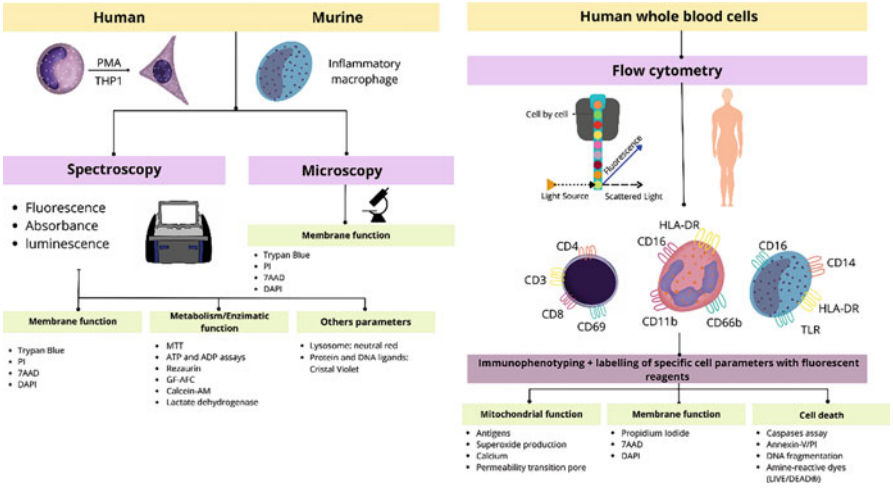

Fig. 4 Methods and reagents (e.g., dyes, substrate, antibodies, etc.) used to assess cytotoxicity based on cell parameters applied to drug discovery of *leishmanicides*.

(**a**) By spectroscopy and microscopy and (**b**) by flow cytometry

Trypan blue is cheap and commonly used in the routine of a laboratory. Counting of blue-stained cells is performed through a hemocytometer or light microscopy (visual inspection). A widely used biomarker to identify dead cells is the quantification of intracellular enzymes in the culture supernatant. One example is the assay to quantify lactate dehydrogenase (LDH), by fluorescence or colorimetric method, a cytoplasmic enzyme that is leaked from inside of dead cells and can be measured directly in the supernatant. Some authors classify the LDH assay as one of the assays based on release of intracellular protein or "based on measurement of activity of cytoplasmic enzymes released by damaged cells" and/or metabolism-based assays (Kumar et al. 2018; Méry et al. 2017).

Methods commonly used as a preliminary protocol for screening compounds are based on the active metabolism of cells. The most cited protocols in literature are the intracellular measurement of adenosine triphosphate (ATP) reduction, colorimetric/fluorometric assay, in cells with compromised membranes (Méry et al. 2017; Riss et al. 2016; Gerets et al. 2009). Another widely used technique is the quantification of metabolization by mitochondrial esterases of resazurin to fluorescent resorufin or metabolization of the tetrazolium salt as MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), into formazan, a colored compound. The activity of succinate dehydrogenase (SDH) in viable cells is also used to monitor cell viability. Further, available assays allow measurement of the constitutive proteases of viable cells after incubation with the cell-permeable fluorogenic substrate GF-AFC (glycylphenylalanyl-aminofluorocoumarin) that is broken into a fluorescent product in living cells. Other lipophilic dyes such as calcein acetoxymethyl (calcein-AM) easily penetrate the cell. Inside of cells, they are hydrolyzed by intracellular esterases generating fluorescent products that are selectively maintained in viable cells (Hu et al. 2021; Gerets et al. 2009). Neutral red is another widely used vital dye that can be incorporated into the lysosome of viable cells (Amorim et al. 2017). Crystal violet is a triarylmethane dye that binds to proteins and DNA of viable cells, allowing quantification of survival and cell proliferation (Hu et al. 2021). It is important to highlight that among these assays, the tetrazolium compound MTT is the preferred method for quantification of the viable cells by mitochondrial metabolism in academic studies for screening purposes (Riss et al. 2016). Nevertheless, all the methods reported above do not allow discrimination of the type of cell death involved, neither is it possible to identify antiproliferative effects of compounds tested (Fig. 4a).

Another in vitro test that rises to investigate the cytotoxicity of compounds is the use of human peripheral blood cells through immunophenotyping, employing the methodology of fluorescence-activated single-cell sorting (FACS), also known as flow cytometry (Fig. 4b). This methodology allows the analysis of several cell components individually and simultaneously such as the cytoplasmic and nuclear membranes, organelles, cytokines, hormones, nuclei, DNA, RNA, and chromosomes. It is possible to perform a phenotypic characterization of the mixed population of peripheral blood cells in the protocol known as immunophenotyping. This procedure permits to analyze individually the cell type of the immune system due to the possibility of specifically marking the different antigens present on them. Each cell has a set of markers on the membrane that allows identification of the leukocyte

population and its subsets and differentiation stages. Monoclonal antibodies labeled with fluorochromes against those markers allow for specific labeling and differentiated counting in the assay (Engel et al. 2015). The markers are so-called clusters of differentiation (CD). For example, CD3 is the differentiation cluster 3 and defines the co-receptor found in all T cells. Monocytes have markers like CD14 and CD11b, and for neutrophils, CD11b, CD16, and CD66b can be used. There are still several markers for the different cell subpopulations. Monoclonal antibodies that recognized these CD are linked to a variety of bright fluorescent reagents such as fluorescein (FITC), phycoerythrin (PE), allophycocyanin (APC), peridinin-chlorophyll-protein (PerCP), and others (Adan et al. 2017).

The cytotoxicity activity and the cell death pathways are also directly quantified by FACS in each population due to the possibility of analyzing multiple cell parameters by flow cytometry, after staining with specific fluorescent dye coupled to the cell or by changes in cell size and granularity parameters. Biochemical and morphological alterations in cells under different cell death processes (apoptosis, necroptosis, autophagy, ferroptosis, pyroptosis, anoikis, lysosome-dependent cell death, NETosis, entotic and mitotic death) can be monitored by specific biomarkers that make it possible to distinguish them. For example, labeling of phosphatidylserine in cells under early apoptosis is performed with annexin-V, associated with PI, to distinguish apoptosis from necrosis. Measurement of PI to quantify DNA fragmentation by FACS allows identifying subdiploid cells in apoptosis, in addition with the measurement of caspase activities and release of cytochrome-c. Intracellular excess of ROS, as the result of different cell death mechanisms, can also be measured using cell-permeant reagent 2′,7′-dichlorofluorescein diacetate (DCFDA), for example. Nonpermeable fluorescent dyes such as 7AAD, DAPI, and PI are used to label DNA preferentially in cells with loss of the selectivity of the membrane under different cell death processes (Hu et al. 2021). Examples of permeable vital dyes are the fluorescent amine-reactive dyes that penetrate dead cell membranes, reacting with free amines from the cytoplasm. Viable cells with intact membranes exclude these dyes and are distinguished from the dead cells by the lower fluorescence intensity. Both cells are further distinguished by flow cytometry analysis based on fluorescence intensity. These dyes are also known as "LIVE/ DEAD® Fixable Dead Cell Stains" and are used also in some studies to characterize cell viability in the immune response to leishmanial drugs (Fig. 4b) (Perfetto et al. 2010; PICK et al. 2004; Ribeiro et al. 2020a).

Proposal of Human Peripheral Leukocytes (HPL) of Whole Blood as a Model to Measure the Cytotoxicity of Leishmanicidal Drugs

The more predictive assay to test potential leishmanicidal drugs must simulate in vitro the infection in immune cells, e.g., the intracellular form of the parasite in monocyte/macrophage and neutrophil cells (Tavares et al. 2018). These cell types should be evaluated about the infection by the parasites and about the impact of the test compounds on their viability, once it is desirable that the chemicals kill the parasite with none or minimal damage to the host cell. There are still challenges to

models that use cells derived from animals or tumor lines, which has encouraged the development of more predictive assays to search for new leishmanicidal drugs.

Why does human whole blood seem to be a good model? The success of any leishmanicidal therapy is dependent on the pharmacological modulation of immunological responses. Consequently, new chemoimmunotherapeutic candidates should be evaluated for their impact on innate and acquired immune response cells as described in Fig. 2 (Rossi and Fasel 2018). The human peripheral blood provides a promising predictive model. The advantage of using peripheral blood is the possibility of immunological functional evaluations such as production of cytokines and chemokines and the complex interactions of the components of the immune response against the test compounds while also evaluating their hazards on the immune system (Villani et al. 2018). Thus, the application of HPL to drug testing is a hopeful model because it includes the variables of individual responses observed in humans. Early identification of potential preclinical safety issues aids the discovery process to select the safest candidates that can proceed in the pipeline, as well as supporting clinical development and toxicology studies until new drugs reach the market (Dobrovolskaia 2015).

Most of the preclinical assays have focused on cytotoxicity studies and the efficacy of compounds, but they neglect their reliance on innate and adaptive immunity, which could be leveraged to improve their efficacy. Promising targets for immunochemotherapy include the different aspects of the immune response such as cytokine regulation, macrophage activation, cell signaling pathways, and costimulatory pathways among cells (Aruleba et al. 2020).

The different adverse effects induced by chemicals represent one potential risk to human security and are associated with changes in the immune balance and reduction or an increase in immune activity. The toxic responses on the immune system are recognized by immune reactions such as allergic responses, cytokine storms, complement activation, contact activation, lower levels of antibodies after vaccination, systemic inflammation, mitochondrial toxicity, and other autoimmune symptoms. These reactions are classified into four main immunotoxic effects such as hypersensitivity, autoimmunity, immune stimulation, and immunosuppression (Zhu and Evans, 2016; Naidenko et al. 2021; Dobrovolskaia and McNeil 2013) (Fig. 5a). These effects are evaluated in the clinic through in vitro assays, and some of them are represented in Fig. 5b.

Thus, it has been advocated that drug discovery platforms and pharmacological studies need to include the assessment of immunotoxicity in their analysis. The goal of these studies at the preclinical level is to identify potential risks before proceeding to the next phases of the pipeline. So, these experimental models must mimic the type of immunotoxicity that can occur in the clinic and be predicted with accuracy in in vitro assays (Aruleba et al. 2020; Villani et al. 2018; Dobrovolskaia 2015). Omics technologies such as genomics, transcriptomics, proteomics, and metabolomics hold promise in providing predictive data on chemical risk in this context that will improve the predictability of new assays (Naidenko et al. 2021). Models for studies about this issue are described on guidelines available online by the WHO (Kimura et al. 2020),

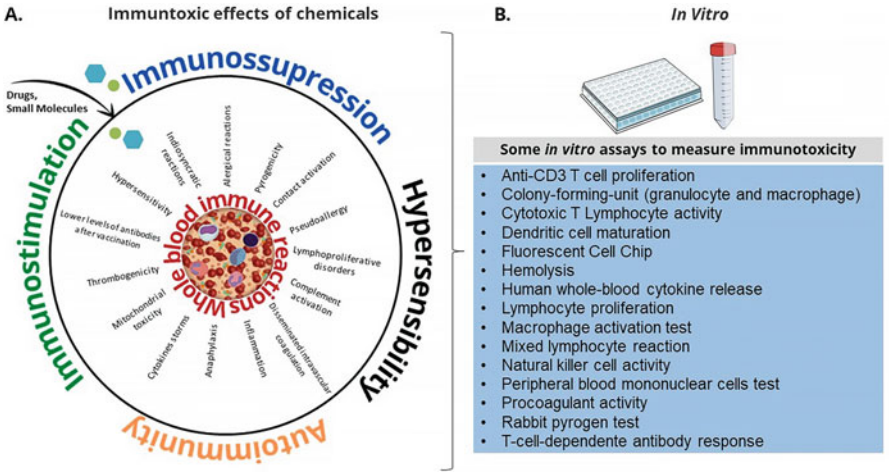

Fig. 5 Examples of immunotoxic effects induced by chemicals and examples of in vitro assays to measure them.

There are four types of undesirable effects of drugs against immune system (immunosuppression, immunostimulation, hypsersensibilization, and autoimmunity) that cause several adverse reactions as displayed in **a** (inside circle, in black). To measure these effects, some examples of the in vitro assays are listed in **b**

Tests for in vitro and in vivo toxicology studies include animals of other species such as mice, rats, rabbits, dogs, swine, guinea pigs, hamsters, rhesus monkeys, sheep, and calves. The cells from animal species have restrictions in predicting the sensitivity/susceptibility to test compounds, as responses to a given stimulus may vary between species (Dobrovolskaia 2015). Animal models have received much criticism for predicting toxicity in humans due to the high failure rates of clinical trials (Bailey et al. 2015). Bailey et al. (2015) evaluated data from 2366 drugs using preclinical studies with three animal species (rat, mouse, and rabbit), as well as in humans. They showed that not observing toxicity in the animal models provides little or quite no evidence that there will be no adverse drug reactions in humans. Similar results were described for dogs and nonhuman primates. These assays are expensive, require large numbers of animals, and raise ethical questions (Kimura et al. 2020). Therefore, there are several drawbacks related to the use of nonhuman models in drug discovery programs.

Specifically, for leishmaniasis, the Chester Beatty hamsters and BALB/c mice are the most referenced in vivo models in the literature (Zulfigar et al. 2017). The use of animal-derived macrophages has led to many discussions, not only because of the differences among species in the response to the compounds but also concerning the principle of the 3Rs (replacement, reduction, and refinement). Since the principle of the 3Rs was implemented to reduce the use of animals and develop new nonanimal-based methods to predict the safety and risk of chemicals to humans, initiatives to create new alternative methods for drug toxicity and safety studies are in evidence. Institutions such as the European Center for the Validation of Alternative Methods

(ECVAM) have promoted scientific meetings aimed at developing new in vitro methods to assess immunotoxicity of drugs using human cells to reduce the use of animal models (Kimura et al. 2020; Bailey et al. 2015; Shi et al. 2021; Zhu and Evans 2016).

To attempt to comply with this demand, Ribeiro et al. (2020b) established an in vitro preclinical predictive assay using as model the human whole blood that contributes to the discovery of new leishmanicidal drugs. This model mimics the natural infection of phagocytes by most *Leishmania* species, allowing to measure simultaneously the cytotoxicity against components of the immune system. As it is well-known, at the initial phase of *Leishmania* infection, recruitment of inflammatory monocytes, macrophages, and neutrophils occurs while the parasite hides, shaping the innate and adaptive immune response to maintain the infection (Rossi and Fasel 2018). The advantage of using peripheral blood is the possibility of functional assessment of complex interactions of components of the immune response against test compounds, as previously described (Villani et al. 2018). Ribeiro's functional protocol provides a better in vitro-in vivo correlation of the immunotoxic potential of the test compounds, allowing to distinguish concurrently between leishmanicidal activity and toxicity to phagocytes, as well as other components of the immune system, in addition to minimizing problems with differences among species, once it uses human cells.

In this context, Ribeiro et al. (2020b) described an ex vivo assay using human whole blood incubated with live *L. braziliensis* that were labeled with different fluorescent dyes to simultaneously assess the leishmanicidal and cytotoxic activity of two drugs used in the clinic by FACS. What novelty did the authors propose? The authors performed an immunophenotyping analysis with a focus on the monocyte population through CD14 labeling with antibody CD14-PE incubated with live parasites labeled with FITC in the presence of amphotericin B and Glucantime®. They performed a multiparametric analysis of the number of fluorescent parasites internalized by CD14 cells to measure leishmanicidal activities of amphotericin B and Glucantime®. The leishmanicidal effect was identified by quantifying the number of internalized forms of the fluorescent parasite (FITC) within the monocyte (CD14 PE) subpopulation. The effect on the cell membrane integrity as indicative of viability was examined by the capability of viable cells to exclude 7AAD stain. Assessment of death was also performed by labeling with annexin-V to identify cells in necrosis and apoptosis (Fig. 6). The interesting aspect about the assay was the possibility of simultaneously evaluating all parameters in the same analyte, under the same experimental variables, differing from conventional methods where the measurement of cell viability and leishmanicidal activities occurs in separated assays/analytes (Zulfigar et al. 2017). The authors demonstrated that the reduction in the number of internalized parasites by monocytes in the presence of amphotericin B and Glucantime® occurred without loss of cell viability, in agreement with the literature. It was identified that the number of leukocytes in cell death by apoptosis and/or necrosis was not significant.

In addition to the quantification of cytokines at the intracellular level, in the same tube, it is possible to quantify the cytokines secreted in the supernatant using other

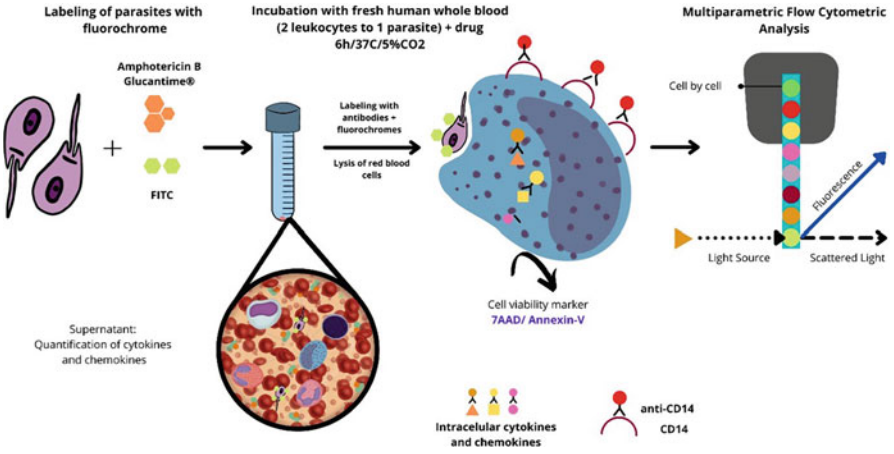

Fig. 6 Scheme of the functional preclinical assay using human peripheral blood to assess leishmanicidal and cytotoxicity activities of drugs using the multiparametric analysis by flow cytometry.

Promastigote forms of leishmania are labeled with fluorochrome (FITC), incubated with fresh human whole blood (1:2 ratio parasite/leukocytes) and drug test. After 6 h of incubation, cells are labeled with antibodies specific for different subpopulations of immune cells (macrophages as an example) and cell viability reagents, followed by erythrocyte lysis and flow cytometry analysis. The assay also allows quantification of cytokines and chemokines at the intracellular level or in the supernatant of the culture using other immunoassays such as CBA, ELISA, and Luminex

immunoassays such as Luminex, ELISA, and Bead Array cytometric system (CBA). In the CBA system, beads with different fluorescence are coated with specific antibodies to evaluate different types of cytokines. This functional assay is indicated as a secondary assay, for selecting the most promising substances such as LEAD and candidate compounds for the next phases of the pipeline. The assay attends to the requirements of the FDA for secondary assays to assess the immunotoxic potential of drugs, which demands functionality measurement of key cell subtypes such as antigen-presenting cells, T cells, and B cells (FDA 2020). In addition, Ribeiro's protocol using immunophenotyping allows investigation of other functional parameters on leukocyte subpopulations such as intracellular chemokines, cytokines, and nitric oxide as previously described (Ribeiro et al. 2020a).

Final Remarks

The contribution of the academe, foundations, and private/governmental research institutes to develop new therapies for the treatment of neglected diseases such as leishmaniasis is well-known. In vitro and in vivo assays demand investments in infrastructure, human resources, reagents, etc., which depend on high-cost investments. For the current strategies for the discovery of new leishmanicidal drugs there

are several challenges and possibilities among the available tests, in terms of cost, execution time, the number of analyzed samples (either for low or high throughput) and the types of in vitro or in vivo models that are more predictive (human) or not (animal). The preclinical multiparametric functional assay by flow cytometry is an excellent option to characterize the cytotoxicity of new immunotherapeutic leishmanicidal drugs. Despite being a high-cost, time-consuming assay, it has advantages such as the possibility of evaluating in vitro immunological modulation and immunotoxicity. Following ethical precepts, whole blood can be obtained "fresh" from healthy donors. It also contributes to reducing the number of animals in research, respecting the 3R regulations. Certainly, concerning conventional assays in which the assessment of the toxicity and leishmanicidal activity is carried out separately, in different plates/tubes, the cytometric assay is a great advance that certainly contributes to the evolution of assays to search for new leishmanicidal agents.

Applications to Other Disease

In this chapter, we highlighted the importance of in vitro assays using human cells to discover new leishmanicidal agents, the challenges of current assays to predict in vivo effects, and the state-of-art regarding drug discovery. The methodology proposed by Ribeiro et al. (2020b) is also suitable for other conditions such as the discovery and development of new drugs for Chagas disease treatment, which is urgent due to the high toxicity and low cure efficacy of the currently available strategies mainly during the chronic phase of this disease. The functional assay is able to measure the internalization of epimastigotes forms of *Trypanosoma cruzi* by human peripheral blood leukocytes from healthy volunteers. The simultaneous analyses by flow cytometry are capable to evaluate the immunocytotoxicity, the anti-*T. cruzi* activity, and the immunomodulatory effect of drugs (benznidazole, ravuconazole, and posaconazole). In addition, it is possible to specifically study components at an intracellular level such as cytokines, chemokines, and NO while also measuring secreted cytokines and chemokines in the supernatant of cultures. This innovative predictive test emerges as a more refined tool for the selection of the most promising compounds which were identified in prospecting programs of new drugs for Chagas disease treatment (Lopes 2016).

Mini-Dictionary of Terms

- Ex vivo assay: assay performed with biological samples (tissue, blood) with minimal alteration of the natural conditions found in vivo.
- LEAD compound: a chemical compound or natural product that has biological activity against a drug target that can be modified to improve specificity to the target, pharmacokinetics and bioavailability, to be evaluated regarding pharmacological functions at preclinical and clinical studies.

- Luminex: immunoassay that allows the detection and quantification of expressed genes and analytes such as growth factors, cytokines, and chemokines, in a single sample.

Key Facts of the Human Whole Blood Model to Measure the Cytotoxicity of Leishmanicidal Drugs

- There is no human vaccine available against *Leishmania* infection, and control is based mainly on chemotherapy, which is limited to the occurrence of drug-resistant parasite strains to the drugs in clinical use.
- Amphotericin B (AmB) is a potent pro-inflammatory stimulant of innate immune cells via the mechanism of oxidative stress in macrophages and intracellular ROS accumulation.
- Miltefosine is currently the only oral leishmanicidal agent and was discovered as a result of a partnership between the WHO, a private company, and researchers from leishmaniasis-endemic countries.
- The drug discovery process should select leads with leishmanicidal action and with synergic action in the immune system. For this reason, it is important to develop methods that allow the evaluation of the immunological response associated with the treatment from the perspective of controlling the infection by the parasite.
- Although there are several in vitro tests to discover new leishmanicidal drugs, many of them fail in preclinical studies because the methods do not accurately predict toxic effects, mainly immunotoxicity.
- The use of human peripheral blood is a promissory option to test the toxicity of leishmanicidal drugs and study the impact of drugs/chemicals in the immune response.
- The multiparametric ex vivo flow cytometry method using human whole blood allows the simultaneous measurement of cytotoxicity (immunotoxicity), leishmanicidal activity, and modulation of immune response of leishmanicidal drugs.

Summary Points

- Immune response regulation is essential for leishmanicidal therapy success. The drug discovery process for a new leishmanicidal agent must focus on immunochemotherapeutic drugs.
- The in vitro and in vivo assays to measure the leishmanicidal activity and cytotoxicity utilize cells derived from animals and humans, which are not so predictive for the preclinical stage and generate failures of several molecules for the follow-up processes in the drug discovery pipeline.

- There are few options of assays to evaluate in vitro immunotoxicity in leishmanicidal drugs during the development phase of the drug discovery process.
- Human peripheral blood is a more predictive preclinical model and accurate to measure the effect of chemicals on the innate and acquired immune response and to overcome the differences between the species.
- The ex vivo multiparametric method using human peripheral blood by flow cytometry allows the simultaneous measurement of the cytotoxicity (immunotoxicity), leishmanicidal activity, and modulation of the immune response in a single analyte.

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Contrast-Induced Nephropathy (CIN) and Biomarkers

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Abstract

Acute kidney injury (AKI) is a sudden loss of kidney function secondary to a variety of reasons. Contrast-induced nephropathy (CIN) is a form of AKI induced following exposure to iodinated contrast media (CM). Its definition varies due to multiple diagnostic criteria but following the Kidney Disease: Improving Global Outcomes (KDIGO) guidelines release, a more unanimous approach has been adopted. Diagnostic tests, invasive cardiac and vascular interventions, as well as aging population contribute to the accretive incidence of CIN. Serum creatinine is the hallmark biomarker that has been utilized for years in order to reflect alterations in renal function, but its kinetics make it a late and non-precise molecule. Emerging serum and urinary biomarkers are eagerly studied in order to yield sensitive and accurate detection, treatment optimization, deflection of unnecessary interventions, and avoidance of detrimental consequences associated with CIN. In this chapter, we attempt to summarize the evidence concerning the available biomarkers and their properties and application to prognosis.

Keywords

Acute kidney injury · Angiography · Biomarkers · Cardiac catheterization · Contrast-induced nephropathy · Computed tomography · Contrast media · Creatinine · Cystatin C · Kidney · Renal insufficiency · Percutaneous coronary intervention

Abbreviations

ACS	Acute coronary syndrome
ADMA	Asymmetric dimethylarginine
ADQI	*Acute Dialysis Quality Initiative*
AKI	Acute kidney injury
AKIN	*Acute Kidney Injury Network*
AUC	*Area under curve*
β_2M	β_2-macroglobulin
CI	Confidence interval
CI-AKI	Contrast-induced acute kidney injury
CIN	*Contrast-induced nephropathy*
CKD	*Chronic kidney disease*
CM	*Contrast media*
CRP	*C-reactive protein*
CVA	*Cerebrovascular accident*
CVD	*Cardiovascular disease*
Cys-C	*Cystatin C*
DKK3	Dickkopf-3
GFR	Glomerular filtration rate
FGF23	Fibroblast growth factor 23
GI	Gastrointestinal

GGT	Gamma glutamyl transferase
GDF-15	Growth differentiation factor 15
kDA	Kilodalton
KDIGO	*Kidney Disease Improving Global Outcomes*
KIM-1	*Kidney injury marker 1*
ICU	Intensive care unit
IGFBP-7	Insulin-like growth factor binding protein-7
IL-6	Interleukin 6
IL-18	Interleukin 18
L-FABP	Liver-Type Fatty-Acid-Binding Protein
NAG	N-Acetyl-b-D-Glucosamine
NF-Kβ	*N*uclear factor kappa-light-chain-enhancer of activated B cells
NGAL	Neutrophil Gelatinase–Associated Lipocalin
NO	Nitric oxide
NPV	Negative predictive value
OR	Odds ratio
PCI	Percutaneous coronary intervention
PPV	Positive predictive value
RIFLE	*Risk, injury, failure, loss, and end stage*
ROC	*R*eceiver operating characteristic
ROS	*Reactive oxygen species*
sCr	*Serum creatinine*
TIMP-2	Tissue inhibitor of metalloproteinases-2
UA	Uric acid

Introduction

Acute Kidney Injury

Acute kidney injury (AKI), previously referred to as acute renal failure, accounts for a rather common clinical condition that designates an abrupt renal dysfunction associated with retention of metabolic waste products and electrolyte disturbances. The incidence of AKI among in-hospital patients may rise up to 20%, contrary to the community, where the incidence is up to 4.3%. AKI represents a complex entity related to increased morbidity and mortality principally for patients with certain risk factors, including dehydration, advanced age, chronic kidney disease, heart failure, liver failure, diabetes mellitus, and anemia. Potential risk factors for AKI are subgrouped into *prerenal* (i.e., hemorrhage, volume depletion, myocardial infarction, sepsis, cirrhosis), *renal* (i.e., rhabdomyolysis, nephrotoxic medications, vasculitis, glomerulonephritis), or *post-renal* (i.e., obstruction).

Historically, the predominant definitions of AKI are based either on the variation of serum creatinine (sCR) level percentage or on the reduction of urine output. In 2002, the *Acute Dialysis Quality Initiative (ADQI) group proposed the RIFLE (risk, injury, failure, loss, end stage) criteria (Table 1) (*Bellomo, Ronco et al. 2004*).*

*Following that definition, in 2007, the Acute Kidney Injury Network (AKIN) group proposed an improved and more sensitive version of the RIFLE criteria in order to address some ambiguities of the initial assessment tool (Table 1) (*Mehta, Kellum et al. 2007*). At present, the Acute Kidney Injury Working Group formed the Kidney Disease Improving Global Outcomes (KDIGO) AKI definition (*KDIGO 2012*) that aimed to unify the various AKI definitions (Table 1).*

Administration of nephrotoxic medications is included among the plethora of AKI causes. Iodinated contrast media (CM) appertain to this category. CM are nonabsorbable agents that, following filtration from the glomerulus, cause a number of pathophysiological effects. Advances in medicine resulted in better quality as well as more precise imaging and therapeutic techniques; hence, there has been an accretive use of iodinated CM for such purposes. Intravenous exposure to CM induces a deleterious iatrogenic form of renal dysfunction. The exerted effect occurs within 24 to 72 hours following exposure. This form of AKI is called contrast-induced nephropathy (CIN) or contrast-induced AKI (CI-AKI).

Table 1 Various AKI definitions

RIFLE criteria			
Stage	sCr	GFR alterations	Urine output
Risk	1.5 × ↑ sCr	> 25% ↓ GFR	<0.5 mL/kg/hr for 6 hours
Injury	2.0 × ↑ sCr	> 50% ↓ GFR	<0.5 mL/kg/hr for 12 hours
Failure	3.0 × ↑ sCr or sCr >4.0 mg/dL with an acute increase of 0.5 mg/dL	>75% ↓ GFR	<0.3 mL/kg/hr for 24 hours or anuria for 12 hours
Loss	Complete loss of kidney function for > 4 weeks		
End-Stage Kidney disease	End stage kidney disease for > 3 months		
AKIN criteria			
Stage	sCr	GFR alterations	Urine output
1	Absolute ↑ in sCr ≥0.3 mg/dL (≥ 26.5 μmol/L) or 1.5 – 2 0 × ↑ in sCr from baseline	–	<0.5 mL/kg/hr for 6 hours
2	>2.0 – 3.0 × ↑ in sCr from baseline	–	<0.5 mL/kg/hr for 12 hours
3	>3.0 ↑ in sCr from baseline or sCr >4.0 mg/dL with an acute increase of 0.5 mg/dL	–	<0.3 mL/kg/hr for 24 hours or anuria for 12 hours
KIDGO criteria			
	Absolute ↑ in sCr ≥0.3 mg/dL (≥ 26.5 μmol/L) within 48 hours or ≥1.5 × ↑ sCr from baseline within the prior 7 days	–	<0.5 mL/kg/hr for 6 hours

Contrast-Induced Nephropathy

CIN represents the third most frequent cause of AKI in hospitalized patients, with an incidence from 5% to 20%, and is associated with significant morbidity and mortality. The most typical pattern of CIN is a transient asymptomatic rise in sCr levels at 24 hours that reach a peak around day 4 and then return to normal within 7 to 10 days. However, persistence of kidney dysfunction may account for up to 18.6% of cases at 3 months, and in such cases, there is an increased mortality risk. Since the term CIN has been introduced more than 50 years ago, its definition has been alternating according to the various definitions of AKI. Therefore, there isn't a strict consensus regarding the definition of CIN in the available literature. Currently, the definition is in accordance with KDIGO criteria as proposed by the ACR Committee on Drugs and Contrast Media (American College of Radiology Committee on Drugs and Contrast Media 2021). Several risk factors are considered responsible for CIN induction. Patient-related factors include age $>$ 65 years, preexisting chronic kidney disease (CKD), anemia, congestive heart failure, diabetes mellitus, peripheral vascular disease, and nephrotoxic medications. Extrinsic risk factors include osmolarity/quantity of CM used, accumulative effect of multiple recent exposures to CM, and route of CM administration.

Route of administration is of particular interest in patients requiring cardiac angiography/percutaneous coronary intervention (PCI) since cardiac catheterization, angiography, and minimally invasive percutaneous coronary or other vascular interventions constitute a large portion of the procedures requiring CM administration (Tsarouhas et al. 2018). Intra-arterial diverges from intravenous administration as CM is injected suprarenally, the concentration reaching kidneys is higher/more abrupt, and the catheterization carries the risk for atherosclerotic emboli dislodgement with subsequent peripheral infarcts. Hence, the incidence of CIN for patients undergoing cardiac catheterization will be invariably higher compared to those who receive CM intravenously. Furthermore, a large number of such patients have longstanding problems or are critically ill. Consequently, the need for distinction of patients at risk for developing CIN is crucial. The two most popular CIN risk assessment tools for patients with cardiac events that require PCI are presented in Fig. 1 (Marenzi et al. 2004; Mehran et al. 2004; Abellas-Sequeiros et al. 2016).

The pathogenesis of CIN remains to a great extent obscure, and the pathophysiology is considered multifactorial. The passage of CM through renal tubules increases tubular osmolarity resulting in energy depletion. CM also incites direct toxicity that may precipitate large vacuole formation up to cellular necrosis secondary to enzymatic activation; cellular DNA fragmentation; downregulation of cell survival pathways, i.e., Akt signaling pathway; and upregulation of cell apoptotic pathways, i.e., NF-kB transcription factor. A major aspect of the pathophysiology of CIN is attributed to reactive oxygen species (ROS) generation that precipitates cellular membrane destruction. Another potential mechanism for CIN is tubular obstruction as a result of tubular protein precipitation or accumulation of apoptotic tubular cells that fall into the lumen causing impaired flow and back leak to the interstitial space. CM may induce cytokine expression or complement activation,

Mehran risk assessment score

- Age ≥ 75 years
- Anaemia (< 39% for men & < 36% for women)
- Diabetes mellitus
- Contrast volume
- Use of intra-aortic balloon pump
- Congestive heart failure (NYHA III / IV)
- Hypotension (SBP < 80mmHg for ≥ 1 hour)
- eGFR 40 to > 60 ml/min/1,73m^2
  - or 20 to 40 ml/min/1,73m^2
  - or < 20 ml/min/1,73m^2
  - or sCr > 1.5 mg/dl

Marenzi CIN risk score

- Age ≥ 75 years
- Anterior myocardial infarction
- Time to reperfusion ≥ 6 hours
- Contrast agent volume ≥ 300ml
- Use of intra-aortic balloon pump

Fig. 1 Risk assessment tools for patients with ACS requiring PCI

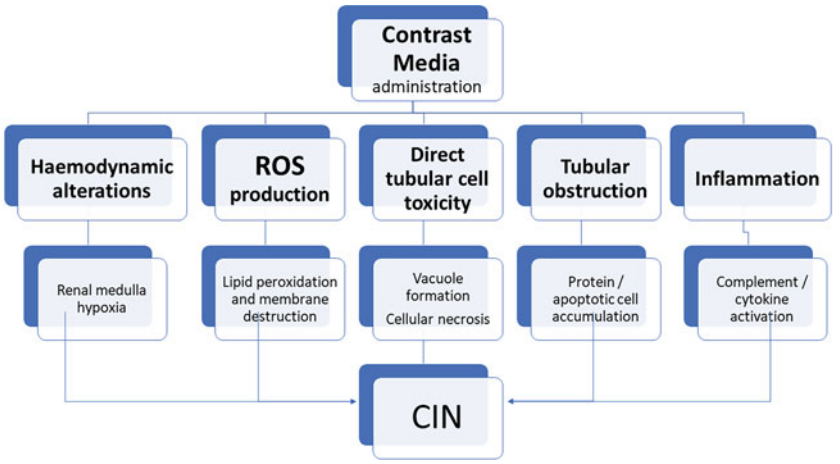

Fig. 2 Summary of the pathophysiological mechanisms that contribute to the development of CIN

resulting in acute tubular necrosis. Finally, the entrance of CM into renal vasculature elicits an unfavorable effect on renal hemodynamics. The initial brief vasodilation is followed by a sustained vasoconstriction due to adenosine and endothelin release. At the same time, CM exerts a direct vasoconstricting effect on smooth muscles and prostaglandin imbalances. These conditions lead to renal medulla ischemia, manifesting as tubular cell injury and necrosis. Simultaneously to the induced ischemia, CM transport through the renal tubules increases energy requirements in the thick ascending limb of Henle's loop, aggravating the medullar hypoxic conditions. A summary of the potential pathophysiological mechanisms that contribute to the development of CIN is presented in Fig. 2 (Mamoulakis et al. 2017; Georgiadis et al. 2020).

Early diagnosis of CIN is vital. In case that complications arise, patients may develop fatal consequences, such as fluid overload, metabolic acidosis, hyperkalemia, permanent loss of renal function, or even death. Currently, sCr is used to determine changes in renal function in everyday clinical practice. However, sCr has some pitfalls due to its kinetics. Consequently, there is a great need for discovering novel more accurate biomarkers that will better assist in prevention, early diagnosis, and optimal management of CIN (Iordache et al. 2020, b).

Biomarkers

Functional Biomarkers

Creatinine

Creatinine is a waste product of muscle and protein metabolism. On a daily basis, 1–2% of human muscle creatine is converted irreversibly to creatinine and is removed mainly by the kidneys through glomerular filtration and tubular secretion. Currently, sCr represents the most frequently used biomarker of renal function – GFR estimation. However, it has several drawbacks; i.e., it is an indirect measurement of renal function; it is affected by age, gender, muscle mass, and nutritional status; and a small portion is reabsorbed in tubules. Additionally, there are medications, which may reduce tubular secretion by inhibiting certain cationic transporters in the proximal convoluted tubules, and most critically, changes in sCr levels do not appear until 50% of renal function is lost. sCr levels react to changes slowly, taking approximately 24 to 72 hours; hence, the chance to detect any change promptly is rather diminished. Despite all these blemishes, sCr still represents the most valid and least biased biomarker that all formal definitions of AKI and CIN adopt.

Cystatin C (Cys-C)

Cys-C is a protein that is literally present in all human tissues as it is produced at a constant rate by all nucleated cells. It inhibits lysosomal enzymes and plays an important role in the preservation of the extracellular structure by inhibiting extracellular cysteine proteases. Cys-C is filtered at the glomerulus and then is absorbed almost completely and metabolized in proximal renal tubular cells. In normal functioning tubular cells, Cys-C levels reflect GFR. Levels are not affected by gender, muscle mass, liver disease, exercise, or inflammation, compared to sCr; hence, it is a more reliable kidney function biomarker. In addition, Cys-C is distributed in the extracellular fluid volume compared to creatinine, which is distributed in the total fluid volume. In other words, creatinine is distributed in three times larger volume; thus, Cys-C increases more promptly. Cys-C has been extensively studied in AKI. Serum Cys-C levels display an age-related rise that indicates its value as an all-cause-related mortality, cardiovascular disease mortality, and cognitive function deterioration biomarker. With regard to CIN, the advantage of the swift rise makes Cys-C an attractive biomarker for early detection and preventive strategies. Alterations in Cys-C levels at 24 hours post CM exposure correlate better

than changes in sCr levels at 48 hours (Kim et al. 2010). It has been shown that serum Cys-C levels increase at 8 hours post-procedure and peak at 24 hours (Malyszko et al. 2009). Among CKD patients, those who develop CIN according to KDIGO definition are better recognized by Cys-C increases $\geq$10% at 24 hours (sensitivity, 100%; specificity, 85.9%; negative predictive value, 100%; positive predictive value, 39.1%) (Briguori et al. 2010).

Microalbuminuria

Microalbuminuria is defined as the presence of 30–300 mg/24 hours of albumin in the urine. Excretion of albumin in the urine is considered a marker of glomerular alterations/dysfunction since it reflects vascular permeability. Detection of albumin in the urine is not a specific biomarker for CIN as it is affected in almost all cases of AKI and CKD as well as in other clinical conditions such as urinary tract infections or bladder tumors. The initial concept that urinary albumin levels are affected by CM administration was corroborated when microalbuminuria improved after N-acetylcysteine administration following CM exposure (Levin et al. 2007). More recently, the value of microalbuminuria in predicting CIN (increase of Cys-C $\geq$ 10%) has been shown in patients submitted to CT coronary angiography. Pre-angiography urine microalbumin levels of 58mg/g creatinine showed a sensitivity and specificity of 74% and 81%, respectively (AUC of 0.84), for predicting CIN (Isobe et al. 2017).

β_2-Microglobulin (β_2M)

β_2M is a molecule that belongs to the major histocompatibility complex. It is filtered by the glomerulus and reabsorbed by renal proximal tubules. In cases of tubular damage, there is reduced reabsorption and subsequent significant alterations in serum and urine levels of β_2M. Prior to coronary angiography, Li et al. showed that serum β_2M had a much higher predictive value of CIN than sCr (AUC, 0.842; $P < 0.001$ vs. AUC, 0.691; $P < 0.001$) at 24 hours and (AUC, 0.937; $P < 0.001$ vs. AUC, 0.908; $P < 0.001$) at 48 hours, respectively. It has also been indicated that baseline serum β_2M levels are an independent predictor for CIN (Li et al. 2015). Uniformly, urine β_2M levels are greatly affected following CM administration in animal studies, showing an up to a 126-fold increase (Kohl et al. 2020). Similar results have been reported in humans but lack statistical significance (Nozue et al. 2010).

Structural Biomarkers

Neutrophil Gelatinase-Associated Lipocalin (NGAL)

NGAL, also referred to as lipocalin-2, is a small peptide of 25 kDA that belongs to the lipocalin superfamily and is bound to the gelatinase of neutrophils. It is produced by neutrophils as well as other sites, such as the kidney, liver, prostate, and respiratory and alimentary tract, as an acute phase protein. NGAL binds to iron-chelating compounds and interrupts bacterial growth. In the kidney, apart from

transportation of lipophilic substances and regulation of iron transportation, NGAL has a growth factor activity regulating growth and differentiation of renal epithelial cells following a renal insult (Connolly et al. 2018). It is produced at the ascending loop of Henle and the collecting ducts and is readily released into the blood and urine. Its value as a predictive biomarker of CIN has been studied in various clinical contexts. In children undergoing coronary angiography, a four- and fivefold increase in plasma levels at 2 hours and 6 hours, respectively, has been reported. Similarly, urine NGAL levels exhibited an eightfold and a tenfold increase at 2 and 6 hours post cardiac catheterization, respectively. A cutoff urine value of 100 ng/ml at 6 hours revealed a sensitivity, specificity, negative predictive value, positive predictive value, and AUC of 90%, 99%, 98%, 90%, and 0.97, respectively, while for similar plasma levels, the respective reported values were 73%, 100%, 96%, 100%, and 0.95 (Hirsch et al. 2007). Similarly for adults developing CIN after PCI, serum NGAL levels increase at 6 hours, peak at 12–24, and decline at 48 hours with very high sensitivity and specificity rates since 6–24 hours (Liao et al. 2019). At the same time, patients undergoing CT angiography experience similar changes, starting at 6 hours (Filiopoulos et al. 2014). In CKD patients, the ability of plasma NGAL levels to predict CIN is satisfactory, showing a significant incremental trend from 2 to 24 hours. Contrary to plasma levels, urine levels fail to show any prognostic value in such patients (Qureshi et al. 2011). In accordance to these findings regarding urine NGAL, poor performance is confirmed for CKD patients by the ANTI-CI-AKI study which concluded that urine NGAL failed to identify CIN in patients with CKD after angiography (Ribitsch et al. 2017).

N-Acetyl-b-D-Glucosamine (NAG)

NAG is a lysosomal enzyme that is found in abundance in the human body. It is produced by the proximal tubular cells, it has high molecular weight, and since it is not filtered by the glomerulus when there is an insult to the proximal tubule, NAG is present in the urine. In the angiography setting, there is evidence that urine NAG increases significantly post CM exposure and peaks earlier than sCr levels, supporting its potential utilization as an early biomarker (Ren et al. 2011).

Kidney Injury Marker 1 (KIM-1)

KIM-1 is a well-studied transmembrane glycoprotein expressed in the epithelial cellular line of the proximal tubule in response to ischemic injury. KIM-1 is absent in healthy human, and its expression is correlated with macrophage infiltration, phagocytosis, cellular dedifferentiation, and tubular obstruction. Evidence supporting the value of KIM-1 as an early CIN biomarker is derived from several studies that demonstrated early significant increase in urine levels from 6 to 48 hours (Akdeniz et al. 2015). In a prospective study of patients requiring angiography +/- intervention, urinary KIM-1 levels at 6 hours showed a sensitivity of 77.8%, a specificity of 82.4%, and an estimated AUC of 0.81 at a cutoff value $>$ 0.43 ng/mg (Wybraniec et al. 2017). On the other hand, BITCOIN study concluded that KIM-1 may provide useful information as a biomarker of early CIN detection but concluded against its use as a prognostic biomarker (Seibert et al. 2020).

Interleukin 18 (IL-18)

IL-18 is a cytokine that belongs to the IL-1 superfamily and is expressed in renal proximal tubules in response to tissue hypoxia. It aggravates inflammatory process by facilitating type 1 processes. It activates the NF-κB pathway and contributes to T-cell differentiation and mast cell/basophil stimulation. A meta-analysis reported that urinary IL-18 levels have a predictive value in AKI (Liu et al. 2013). An increase of >25% in urinary IL-18 levels, at 24 hours after CM exposure, predicts a significant risk for developing CIN (OR 10.71; 95% CI = 1.76–65.24). Similarly, it has been reported that urine IL-18 may predict CIN 24 hours earlier to sCr (sensitivity, specificity, and AUC of 69.2%, 74.1%, and 74.9% at a cutoff point 15.8 pg/ml) (Ling et al. 2008). More recent data suggest that urine IL-18 levels increase as early as 6–12 hours, showing a sensitivity, specificity, and AUC of 87.5%, 62.2%, and 0.811 at a cutoff level of > 815.61 pg/ml, respectively, for detecting CIN at 12 hours post-procedure (He et al. 2014). Serum IL-18 levels at 24 hours post CM administration were also reported to be higher in patients with CIN (Zdziechowska et al. 2020).

Liver-Type Fatty-Acid-Binding Protein (L-FABP)

FABPs are intracellular proteins that act as transporters of free unsaturated fatty acids to the mitochondria or peroxisomes assisting in cellular metabolism. Additionally, they bind to fatty acids produced by lipid peroxidation. Two types of FABPs are detected in the kidney, the L-type which is localized in proximal tubules and the H-type which is isolated in distal tubules. AKI induces L-FABP production, likely secondary to hypoxia, ROS production, and hemodynamic changes as well as the increased filtered fatty acids that are bound to albumin, associated with proteinuria caused by renal dysfunction. Several studies have shown that L-FABP levels increase between 4 and 24 hours, peak at 12 hours, and remain elevated 48 hours following CM exposure in patients who will develop CIN (Bachorzewska-Gajewska et al. 2009; Malyszko et al. 2009). It has also been suggested that in humans who will receive CM, high urinary L-FABP levels prior to administration may predict CIN (Nakamura et al. 2006). In preexisting chronic kidney disease, L-FABP levels of 24.5 μg/g creatinine, prior to CM delivery, detected CIN development with 82% sensitivity as well as 69% specificity. For such levels, the AUC was 0.7 and in a univariate analysis the OR was 8.99 [95% CI =3.34–27.0, $p<0.001$], indicating that L-FABP is an independent predictor of CIN (Manabe et al. 2012). Contrary to urinary L-FABP, serum levels appear to have equivocal value as studies do not provide reproducible results (Connolly et al. 2018; Zdziechowska et al. 2020).

Midkine

Midkine is a heparin binding growth factor with various cellular roles, i.e., growth, migration, survival, and apoptosis regulation. It is expressed in proximal and distal renal tubular cells, and its expression is associated with oxidative damage via the hypoxia- inducible factor-1a pathway. Its potential value as biomarker has been assessed in patients undergoing cardiac angiography. Following PCI, serum midkine

levels were significantly increased at 2, 4, and 8 hours in patients developing CIN (Malyszko et al. 2015).

Dickkopf-3 (DKK3)

DKK3 has been recently introduced in clinical practice as a stress-induced biomarker deriving from renal tubular cells. It has been associated with interstitial fibrosis via the Wnt/β-catenin signaling pathway, and thus it has been utilized for CKD progression assessment. DKK3 was evaluated in AKI setting very recently in a cohort of patients submitted to elective cardiac surgery, who were reported to show a significantly increased risk for developing AKI, persistent renal dysfunction, and need for hemodialysis at urinary DKK3/creatinine ratio of > 471 pg/mg (Schunk et al. 2019). Furthermore, DKK3 was validated as a biomarker for CIN prediction in the coronary angiography setting. Patients who developed CIN exhibited an at least 3.8-fold higher DKK3/creatinine ratio pre-procedurally compared to those who did not develop CIN (sensitivity, specificity, positive predictive value, negative predictive value, and AUC of 47.4%, 72.4%, 8.0%, 96.1%, and 0.61, respectively, at a cutoff value of 1.7 pg/mg creatinine) (Seibert et al. 2021).

Miscellaneous Biomarkers for CIN

Gamma-Glutamyl Transferase (GGT)

GGT is principally present in the liver, pancreatic, and kidney tissue. GGT is predominantly utilized as a test for the extracellular metabolism of glutathione. Glutathione plays an essential role in the endogenous antioxidant protection system. The oxidative stress induced by CIN as well as the potential endothelial vascular damage alongside with the inflammation may be the association between CIN and GGT. Levels of GGT> 26.5 U/L may assist in CIN prediction with a sensitivity of 70% and specificity of 60% (Oksuz et al. 2015).

Homocysteine

Homocysteine is a common amino acid that acts as a potent excitatory neurotransmitter leading to oxidative stress, reduced NO levels, endothelial damage, and cellular death. These effects resemble many aspects of CIN pathophysiology. Several studies evaluated pre-procedural hyper-homocysteinemia and indicated that increased levels of serum homocysteine is an independent predictor for CIN, similarly to Cys-C and superior to sCr (Barbieri, Verdoia et al. 2015b, Li et al. 2015).

Procalcitonin

Procalcitonin is a glycoprotein that consists of 116 amino acids and is the precursor of calcitonin, which is involved in calcium homeostasis. Procalcitonin has demonstrated its value as a biomarker in systemic inflammation conditions. As predictive biomarker for CIN, procalcitonin has been evaluated in patients requiring PCI, and the results demonstrated a cutoff approximate value of 65 pg/mL for CIN prediction

(Kurtul et al. 2015). Although when procalcitonin was studied alone, the results demonstrated very high sensitivity and very low specificity, 96.4% and 37.8%, respectively, and the combination of procalcitonin with high-sensitivity C-reactive protein improved the sensitivity to 54.6%, demonstrating that the pre-procedural raised inflammatory markers may assist in the prediction of CIN (Gu et al. 2019).

Hepcidin

Hepcidin introduces a peptide that is involved in iron regulatory processes. Its main production site is the liver, but it also appears as an intrinsic protein produced by epithelial cells in the kidney (Kulaksiz et al. 2005). The responsible encoding gene is mainly affected by iron accumulation, inflammation, and hypoxia. As these conditions are tightly related with CIN, hepcidin has been evaluated as a predictive biomarker in patients requiring elective PCI. It has been shown that serum hepcidin levels rise significantly as early as 4 hours compared to its urinary levels that demonstrate a significant fall at 8 hours after the intervention in patients developing CIN (Malyszko et al. 2019).

G1 Cell-Cycle Arrest Proteins

Tissue inhibitor of metalloproteinases-2 (TIMP-2) is a natural inhibitor of the peptidases that are involved in the extracellular matrix degradation. It also possesses the ability to directly inhibit endothelial proliferation. Insulin-like growth factor binding protein-7 (IGFBP-7) is a peptide that modulates the availability of insulin-like growth factors in tissue with an effect on cellular growth, proliferation, apoptosis, and angiogenesis. These two biomarkers were evaluated in the critically ill setting of patients admitted to the intensive care unit. A combination of the two biomarkers exhibited an AUC of 0.8 for development of AKI and outperformed other well-established biomarkers such as NGAL, KIM-1, Cys-C, and L-FABP (Kashani et al. 2013). However, when these biomarkers were testing for CIN, results were inconclusive.

Semaphorin 3A

Semaphorin is a peptide that belongs to the semaphoring family and acts as chemoattractive or chemorepellent agent mediating neuronal pattern development. It is secreted by neurons and perineural tissue in order to guide axonal architecture. Semaphorin is also found to be expressed in the glomerulus, podocytes, and collecting tubules. Its mechanism of action is through signaling receptors plexin A1 or A3 and binding neuropilin-1 receptor. Semaphorin's 3A value as a predictive biomarker for AKI was assessed in patients undergoing cardiopulmonary bypass. It was shown that urine levels rose as early as 2 hours and peaked at 6 hours with AUCs of 0.88 and 0.81, respectively (Jayakumar et al. 2013). Its potential value was further assessed and verified in patients with stable angina requiring routine coronary angiography. Urine semaphorin 3A levels showed a sensitivity, specificity, and AUC at 2 and at 6 hours of 94%, 75%, 0.858 and 0.765 at a cutoff value of 389.5 pg/mg creatinine for predicting CIN (Ning et al. 2018).

Fibroblast Growth Factor 23 (FGF23)

FGF23 is a protein that assists in phosphate and vitamin D regulation and metabolism. The secretion of FGF23 is mediated by calcitriol and is secreted by osteocytes. It acts by reducing the expression of renal Na (+)/phosphate (Pi) cotransporter in the proximal tubule, minimizing calcium reabsorption and increasing phosphate excretion. Similar to other biomarkers, FGF23 levels were found to be raised in the AKI setting. Thus, its potential value as a diagnostic biomarker for CIN was tested again in patients submitted to angioplasty. Serum FGF23 levels displayed a remarkable rise within 48 hours following the intervention in the CIN group (sensitivity, specificity, and AUC at 24 hours of 73.3%, 87.6%, and 0.814, respectively, at a cutoff point of 20%) (Li et al. 2018).

Growth Differentiation Factor 15 (GDF-15)

GDF-15 belongs to the transforming growth factor family, and it represents a stress responsive cytokine and is poorly expressed in tissue under normal circumstances. In the presence of inflammation, oxidative stress injury, or endothelial dysfunction, GDF-15 expression is increased. It is vastly found in cardiac tissue, adipocytes, and vascular and endothelial cells. There is evidence that GDF-15 assists in tissue protection through multiple pathways, i.e., JNK, EGFR, PI3K, and AKT (Adela and Banerjee 2015). It normally indicates disease progression, and due to the fact that its activation process shares similarities with the pathogenesis of CIN, it has been investigated as a potential biomarker for predicting CIN. Available data indicate that GDF-15 levels show promising results in CIN but need further verification in larger cohorts (AUC: 0.744, which was poorer compared to creatinine's performance but combined with sCr revealed better predictive value than sCr alone) (Sun et al. 2018).

Uric Acid (UA)

Uric acid is the product of the metabolism of purines. It is a normal component of human blood and urine. Elevated levels of UA are associated with renal dysfunction by inducing nitric oxide inhibition, endothelin-1 and renin-angiotensin stimulation, urate crystal formation, ROS formation, and pro-inflammatory effects. Multiple studies have investigated the value of UA as a potential biomarker for CIN, especially in patients undergoing angiography showing an association between elevated UA and CIN (OR 1.42 for UA levels of $\geq$ 7 mg/dl (Barbieri, Verdoia et al. 2015a) and OR 1.76 for UA levels of $\geq$ 6.7 mg/dl (Mandurino-Mirizzi et al. 2021)).

Adiponectin

Adiponectin is a polypeptide that provides a regulatory mechanism of metabolic processes such as fatty acid breakdown and glucose control. It also acts as anti-inflammatory agent on vascular endothelial cells. In animal models, adiponectin has illustrated a significant role in the improvement of podocyte dysfunction and permeability to proteins through oxidative stress reduction (Sharma et al. 2008). Hence,

a possible association with CIN has been sought in patients with coronary artery disease requiring PCI. An optimal cutoff value of 12.24 ng/ml demonstrated a sensitivity of 68.4%, specificity of 76.7%, and an AUC of 0.720. Adiponectin levels $>$ 12.24 ng/ml have been shown to be a significant predictor of CIN (OR 5.071) (Zhang et al. 2019).

Glucose

Elevated glucose levels are anticipated in critically ill patients or patients with acute myocardial infarction. This observation is highly likely to be related to stress-induced cytokine and catecholamine expression, or it may postulate insulin resistance (Hafidh et al. 2007). Although diabetes is a risk factor for CIN, there was no significant correlation between glucose levels and CIN in patients with diabetes and acute myocardial infarction requiring angiography. In contrast, high pre-interventional glucose levels in patients without diabetes designated a strong association with CIN. Incremental risk was observed with increasing pre-procedural glucose levels. For levels 110 to $<$ 140 mg/dl, OR was 1.31; 140 to $<$170 mg/dl, OR was 1.51; 170 to $<$200 mg/dl, OR was 1.58; and $\geq$ 200 mg/dl, OR was 2.14 (Stolker et al. 2010). A pre-procedural glycemic value of $>$124 mg/dl added to the Mehran score significantly increased the sensitivity and specificity of the modified scoring system (GlyMehr), especially in nondiabetics (GlyMehr; AUC 0.787, Mehran; AUC 0.706) (Nusca et al. 2021).

Copeptin

Copeptin is a neuropeptide that originates from the c-terminus of the pre-prohormone of the antidiuretic hormone. It is thought to be a marker of intense stress or hemodynamically unstable conditions and has been associated with cardiovascular and kidney disease. As CIN is associated with stress, the hypothesis of possible value of copeptin as predictive biomarker in early diagnosis of CIN has been recently investigated in patients with acute coronary syndrome. Copeptin levels were found to be significantly elevated in patients with CIN. Pre-procedural levels of copeptin of $>$7.72pmol/l produced an AUC of 0.720 with sensitivity and specificity of 73.9% and 73.7%, respectively. Thus, elevated levels need to be carefully judged, and appropriate preventive measures should be initiated (Yildirim and Cabbar 2019).

Circulating microRNA (miRNA)

miRNAs are endogenous, noncoding molecules that have multiple molecular actions. They act as mediators of survival/proliferation and moderate cell signaling networks. They are implicated in kidney development, functionality, and renal disease. Some of them are kidney specific and may serve as biomarkers for renal disease, but most of them have been assessed in animal models. Only miRNA-30a, miRNA-30c, miRNA-30e, and miRNA-188 have been verified in humans. miRNA-30a, miRNA-30e, and miRNA-188 have been implicated in CIN. miRNA-188 and miRNA-30a share a common mechanism of action, the MAPK-JNK/p38 pathway, promoting CM-induced apoptosis (Toruan et al. 2020). miRNA-188 with a fold

change >1.343 yielded an AUC 0.784 with sensitivity of 52.1% and specificity of 93.0%. When a fold change of 1.405 in miRNA-30a was observed, the AUC increased to 0.802 with sensitivity of 56.3% and specificity of 94.4%. Likewise, for a fold change of 1.428 in miRNA-30e levels, the AUC was 0.805 [0.733–0.858] with sensitivity of 64.8% and specificity of 93.0%. When all these molecules exceed the cutoff points, the specificity increased to 97.2%. Similarly, these molecules have been validated against Mehran score revealing a positive correlation between elevated miRNA levels and Mehran score $\geq$ 6 (Sun et al. 2016). Their levels are significantly elevated within 4–6 hours. Another study assessed miRNA-30c in addition to the aforementioned. At a cutoff level of 67.5 fmol/L, the AUC was 0.888, with 98.9% specificity and 65.2% sensitivity, and the positive predictive value was 93.1% (Gutiérrez-Escolano et al. 2015). The advantage of these molecules is their stability in serum and urine; thus, they are ideal for diagnosis and follow-up.

C-Reactive Protein (CRP)

CRP is an acute phase protein, deriving from the liver, and rises in response to inflammation, especially IL-6 production. CRP mechanism of action is to bind to the phosphocholine present on apoptotic cell or bacteria surfaces and activate the complement process in order to stimulate macrophage action and phagocytosis. Inflammation is a well-established risk factor for CIN development. CRP impairs tubular epithelium cell regeneration and promotes AKI by inhibiting CDK2/cyclin E via Smad3 pathway (Tang et al. 2014; Lai et al. 2016). The analysis of a large cohort of patients who underwent coronary intervention indicated that elevated pre-procedural CRP levels were significantly higher in patients who developed CIN (Gao et al. 2011). Based on evidence that CRP, both pre and post-procedural levels, is a useful biomarker, a new scoring system was introduced: the Athens CIN score. It incorporated four cofounders, admission CRP, age, glomerular filtration rate, and ejection fraction, and conveyed good accuracy in predicting CIN (Lazaros et al. 2016). In a neurosurgical setting, incorporating diagnostic angiography, hs-CRP >5 appeared to be a risk factor for CIN (AUC 0.749%) (Kim and Jo 2020).

Asymmetric Dimethylarginine (ADMA)

ADMA is an endogenous inhibitor of all NO synthases. At the same time, it is known to interfere with various other processes such as inflammation, vasodilation, insulin sensitization, antioxidant action, as well as migration, differentiation, and functionality of progenitor cells (Thum et al. 2005). Preclinical studies have indicated that CM exposure results in significant elevation of ADMA levels both immediately after exposure and at 24 hours (Mamoulakis et al. 2019). The preclinical outcomes were confirmed in patients with stable angina pectoris that received CM in an angiography setting. It is indicated that for ADMA levels > 124.7 ng/ml, AUC was 0.780, with sensitivity and specificity of 80% and 76%, respectively, allowing for the assumption that ADMA is an independent predictor for the development of CIN (Özgür Günebakmaz et al. 2013).

Applications to Prognosis

In this section, we will refer to the biomarkers and their potential as they have been established with the strongest level of evidence. There is available evidence from two meta-analyses evaluating **Cys-C** vs. sCr with similar results. The first meta-analysis concluded that pooled diagnostic OR for predicting CIN was 24.2 (95% CI: 7.9–73.7) with a pooled sensitivity and specificity of 83% and 82%, respectively. The optimal time for measurement is likely at 24 hours with OR 53.8 (95% CI: 13.6–213.3), pooled sensitivity of 88%, specificity of 88%, and AUC of 0.93 compared to OR 7.6 (95% CI: 1.3–44.1) with 81% sensitivity, 96% specificity, and AUC of 0.75 when the measurement was obtained in less than 24 hours (Chen et al. 2020). Another meta-analysis concluded that the pooled sensitivity, specificity, and AUC were 87%, 86%, and 0.93, respectively, confirming the diagnostic value of Cys-C in CIN (He et al. 2020). Regarding **NGAL**, the most robust evidence derives from a meta-analysis concluding that the pooled diagnostic OR of plasma NGAL for predicting CIN was 25 (95%CI: 6–108) with a pooled sensitivity, specificity, and AUR of 86%, 80%, and 0.90, respectively. Likewise, for urine NGAL, the diagnostic OR was 22 (95%CI: 8–64) and the AUC was 0.89. The same analysis suggested that plasma levels have superior diagnostic accuracy within 6 hours of CM exposure, but after that, urine NGAL may show better accuracy (He et al. 2020). For urine ***KIM-1***, the most recent and sound evidence regarding its predictive value following coronary intervention derives from a meta-analysis calculating a pooled sensitivity of 84% and specificity of 78%. The AUC was 0.88, supporting that urine KIM-1 is an adequate biomarker for CIN detection earlier than sCr (Li et al. 2020). **Uric acid** does not have a concrete position in CIN diagnosis. Nevertheless, a recent systematic review and meta-analysis concluded that hyperuricemia is associated with the incidence of CIN suggesting that UA may be utilized as a biomarker to select high-risk patients and adopt preventive strategies (Zhang et al. 2019). Last but not least, appertaining to **CRP**, in PCI or coronary artery bypass grafting setting, a systematic review and meta-analysis has provided a strong link between high pre-procedural CRP levels and higher CIN incidence (Wu et al. 2021).

Applications to Other Diseases or Conditions

Above and beyond their merit in CIN diagnosis, some biomarkers are not exclusive to renal disease and exhibit predictive or prognostic properties on various other medical entities. Table 2 summarizes their application to clinical conditions other than any form of AKI.

Conclusion

In view of the expanding usage of iodinated CM for diagnostic and therapeutic purposes alongside with the progressively aging population, the risk for developing CIN is becoming adversely higher. Development and validation of biomarkers with

Table 2 Application to other diseases or conditions

Biomarker	Disease/condition	Biomarker	Disease/condition
ADMA	Insulin resistance to skeletal muscle leading to diabetes	***IGFBP7***	–
Adiponectin	Metabolic syndrome	*IL-18*	Still's disease
α_2 **microglobulin**	CMV infection, Multiple myeloma	***KIM-1***	–
Copeptin	Liver cirrhosis	***L-FABP***	Liver cirrhosis, Acute on chronic liver failure
CRP	CVD, Diabetes, Parkinson's disease, Macular degeneration	***Microatbuminuria***	CVD
Creatinine	–	***Midkine***	Lung–GI–Bladder cancer
Cys-C	CVD	***miRNA***	Cancer, Sepsis, CVD, Alzheimer's disease
Dickkopf-3	Systemic Lupus Erythematosus, Chronic Graft versus Host disease on haemopoietic cell transplant	***NAG***	CVD
FGF-23	Fibrosis, Heart failure	***NGAL***	ACS, Heart failure, CVA
GDF-15	Heart failure Mitochondrial respiratory chain deficiency	***Procalcitonin***	Sepsis
GGT	CVD, Liver injury-failure, Hepatocellular cancer	***Semaphorin 3A***	Systemic sclerosis
Glucose	Diabetes	***TIMP2***	–
Hepcidin	Iron deficiency anemia, Obstructive sleep apnea, Insulin resistance	***Uric acid***	Oxidative stress in heart failure, Metabolic syndrome
Homocysteine	Neurodegenerative disease, CVD, Cancer		

specific characteristics, such as cost-effectiveness, noninvasiveness, quantifiability, reproducibility, high sensitivity, and specificity, is imperative in order to permit risk stratification and monitoring. Taking into account that the available interventions for managing CIN are limited, patients at high risk should be identified to minimize morbidity and mortality. Currently sCr is the only approved biomarker for defining, detecting, and following up CIN, but it is far from being the ideal one. There is a large panel of biomarkers that have been studied, but only few of them have attained valuable evidence-based distinction. The most promising and better studied biomarkers are Cys-C, NGAL, L-FABP, KIM-1, and IL-18. However, they need further validation in larger, multicenter, randomized controlled trials as we are currently lacking sound evidence regarding the time of measurement, the optimal cutoff values, the potential benefit from application in specific patient groups (patients with diabetes or CKD, etc.), and the combined accuracy of multi-marker panels.

Mini-Dictionary of Terms

- ***NF-kB transcription factor pathway.*** An abundant transcription factor that is located inactive in the cytosol. Following extracellular signals, it gets into the nucleus and initiates DNA transcription.
- ***Lipid peroxidation****. The process that imbalanced oxidative stress induces destruction of cellular membranes resulting into apoptosis and cellular death.*
- ***Peptidase****. It refers to a group of enzymes that break down proteins.*
- ***Reactive oxygen species.*** Regular by-products of the oxygen metabolism that participate in cellular communication and homeostasis.
- ***Hypoxia-inducible factor-1a pathway****.* In hypoxic conditions, as a response to stress, HIF-1a induces the expression of several factors that assist the hypoxic cells into changing their metabolism. This helps to maintain the cellular activity despite the lack of oxygen.

Key Facts of CIN

- Contrast-induced nephropathy affects 5–20% of hospitalized patients receiving CM.
- It is associated with metabolic acidosis and significant biochemical abnormalities.
- Its pathophysiology is partly understood, but it is attributed mainly to ischemic injury due to hemodynamic changes, interstitial inflammation, ROS formation, and direct toxicity.
- sCr has been the benchmark biomarker for the diagnosis and prognosis albeit it lacks immediacy and specificity.
- Cys-C is a rapid biomarker with 100% negative predictive value and pooled sensitivity of 87% and specificity of 86%.
- Plasma NGAL levels have superior diagnostic accuracy within 6 hours of CM exposure, but after that, urine NGAL may show better accuracy as biomarker.
- Urine NGAL does not perform well in patients with preexisting CKD.
- KIM-1 is an adequate biomarker for CIN detection earlier than using sCr.
- When pre-procedural glucose levels are combined with Mehran score, sensitivity and specificity is increased.
- High inflammatory markers like CRP are tightly associated with higher CIN incidence.

Summary Points for CIN

- CIN is a serious iatrogenic form of AKI following administration of iodinated CM during diagnostic and therapeutic procedures.
- The most critical risk factors for developing CIN are CKD, diabetes mellitus, and nephrotoxic medications.
- Route, volume, osmolarity, and dose intervals are principal extrinsic risk factors for developing CIN.

- The pathophysiology of CIN is multifactorial; thus, the diagnosis cannot be focused on a single pathway.
- KDIGO criteria are the recommended parameters for defining and diagnosing CIN as they were developed to assist in preventing morbidity and mortality of AKI.
- Ideal biomarkers should be noninvasive, reproducible, cost-effective, quick, sensitive, and specific allowing for early diagnosis, prediction, and monitoring.

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Lead and Aquatic Ecosystems, Biomarkers, and Implications for Humankind

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Abstract

Lead, a highly toxic metal, is present in all the biotic and abiotic environmental components. Its continuous use for several industrial and domestic purposes has caused rise in its levels, thereby posing serious threats to animal and human health. In aquatic ecosystems, fish are highly susceptible to the deleterious effects of lead exposure. This toxicity is induced mainly by bioaccumulation in tissues which then causes oxidative stress due to the production of reactive oxygen species. In addition to this, lead being a divalent cation can surrogate for calcium at multiple levels affecting various cell signaling pathways. Fish can directly affect humans via food intake. Disorders of different body systems in humans and the role of inflammation due to lead exposure have been proven by various studies. These studies indicate that lead exposure may cause respiratory, neurologic, digestive, cardiovascular, and urinary diseases. Both acute and chronic lead exposure can cause many detrimental systematic effects including hypertension, frank anemia, cognitive deficits, infertility, immune imbalances, delayed skeletal and deciduous dental development, vitamin D deficiency, and gastrointestinal effects. The molecular basis of lead exposure resulting in various systemic effects is being extensively explored. Therefore, the aim of this review is to examine the various harmful effects of lead exposure on aquatic organisms and humans, including bioaccumulation, oxidative stress, and immune responses because of lead-induced toxicity.

Keywords

Lead toxicity · Aquatic ecosystem · Humankind · Blood lead level · Reactive oxygen species · Respiratory · Neurologic · Digestive · Cardiovascular · Kidney · Oxidative stress · Inflammation · Biomarkers

Abbreviations

ALA	Aminolevulinic acid
ALAD	Delta aminolevulinic acid dehydratase
ALAS	Delta aminolevulinic acid synthase
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANS	Autonomic nervous system
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BFCOD	7-Benzyloxy-4-trifluoromethyl-coumarin-*O*-debenzyloxylase
BLL	Blood lead level
BP	Blood pressure
CAM-II	Calmodulin-II
CAT	Catalase
CNS	Central nervous system

CREB	cAMP response element-binding protein
CVD	Cardiovascular disease
EROD	7-Ethoxyresorufin-*O*-deethylase
GGT	Gamma-glutamyl transferase
GI tract	Gastrointestinal tract
KIM1	Kidney injury molecule 1
LPO	Lipid peroxidation
MDA	Malondialdehyde
NMDA-r	*N*-methyl-D-aspartate receptor
nNOS	Neuronal nitric oxide synthase
PKC	Protein kinase C
ROS	Reactive oxygen species
SOD	Superoxide dismutase
UGT	UDP-glucuronosyl transferase
αGST	Alpha-glutathione *S*-transferase

Introduction

Lead is a natural component which makes up only about 0.0013% of the earth's crust. It frequently exists at trace levels in the environment (Litasov and Shatskiy 2016). The color of the metal is bluish gray, and it has a soft luster (Tchounwou et al. 2012). The symbol of lead is Pb which is derived from the Latin origin "plumbum" meaning soft metals. The imperative physical properties of lead like its softness, malleability, ductility, resistance to erosion, and conductivity of electricity make it difficult to give up its use (Ab Latif Wani and Usmani 2015). The most important ore of lead is galena. The other notable ores are the weathered products of galena and are generally found nearer to the surface of the earth's crust (Dube 2006) (Table 1). Lead is one of the seven metals of antiquity which was explored by humans, and the maneuver of lead predates those of metals like copper and bronze, dated back to 3000 BC.

Lead (atomic number, 82; relative atomic mass, 207.2) is a member of Group 14 (IVA) of the periodic table. Natural lead is a mixture of four stable isotopes, ^{208}Pb (51–53%), ^{206}Pb (23.5–27%), ^{207}Pb (20.5–23%), and ^{204}Pb (1.35–1.5%). Lead isotopes are the stable decay product of three naturally radioactive elements: ^{206}Pb from uranium, ^{207}Pb from actinium, and ^{208}Pb from thorium. Lead exists in three

Table 1 Different ores of lead (Pb)

Sl. No.	Name of ore	Chemical name	Chemical formula
1.	Galena	Lead sulfide	PbS
2.	Cerussite	Lead carbonate	PbCO3
3.	Anglesite	Lead sulfate	PbSO4
4.	Minium	Lead oxide	Pb_3O_4
5.	Pyromorphite	Lead chlorophosphite	$Pb_5(PO_4)_3Cl$

oxidation states: Pb (0), the metal; Pb (II); and Pb (IV). Lead is predominantly found in the environment in the +2 state. Pb (IV) is only formed under extremely oxidizing conditions, and thus inorganic Pb (IV) compounds are not found under ordinary environmental conditions. While organolead (II) compounds are known, organolead chemistry is dominated by the tetravalent (+4) oxidation state. Metallic lead, Pb (0), exists in nature; however, its occurrence is rare (Acharya 2013).

Sources of Lead Exposure

Albeit lead occurs naturally in the environment, anthropogenic activities such as fossil fuel burning, mining, and manufacturing accord to the release of high lead concentrations (Fig. 1). Lead has many different industrial, agricultural, and domestic applications. It is presently used in the manufacturing of lead-acid batteries, ammunitions, metal products like solder and pipes, and devices to shield X-rays (Tchounwou et al. 2012). Additionally, other sources of Pb are exhaust fumes of automobiles, chimneys of factories using Pb, effluent from the storage battery, smelting, Pb ores, metal plating and finishing operations, fertilizers, pesticides, additives in pigments, and gasoline (Eick et al. 1999). Lead is a toxic heavy metal which leads to major environmental pollution because of its environmental

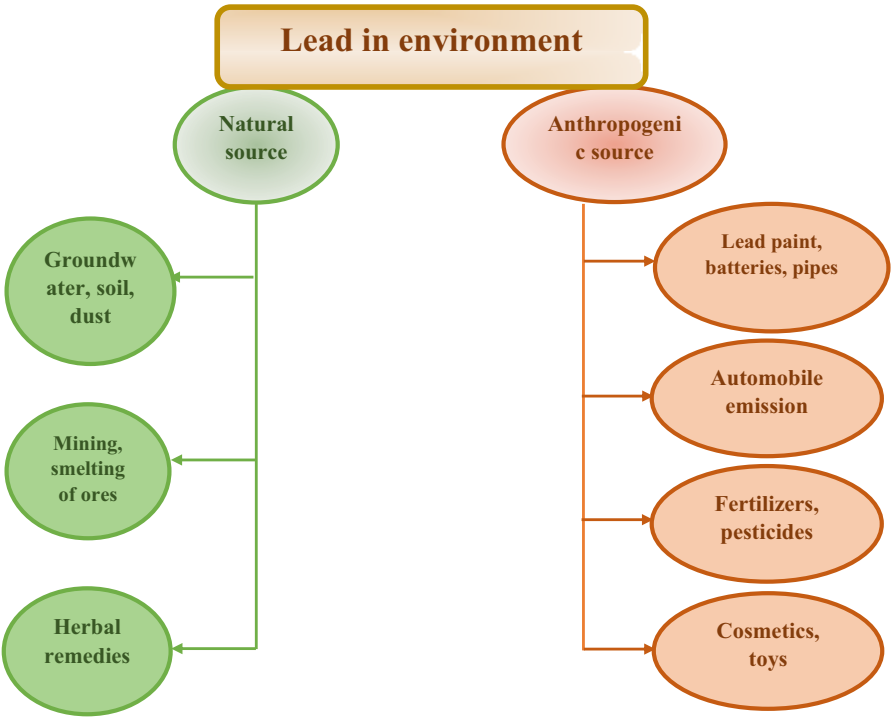

Fig. 1 Sources of lead (Pb) in the environment

persistence and transportability. The nonbiodegradable nature of lead is the fundamental cause of its persistence in the environment (Mitra et al. 2017).

Lead as a Toxic Pollutant

Both occupational and geological exposures have resulted in lead being a serious concern in several developing and industrializing countries. The natural and anthropogenic activities add to the raised levels of toxic Pb in the inert environment which then tend to bioaccumulate. Pb contamination in the environment is very marked as compared to the other nonessential elements (Flegal and Smith 1992). Significant exposure to lead is an environmental risk to the optimal health and physical development in young children that affects all the socioeconomic groups. Globally, the substantial processing of Pb ores is gauged to have released approximately 300 million tonnes of free Pb into the environment over a period of five millennia, mostly within the past 500 years (Tong et al. 2000).

Prior to 1970, blood lead levels more than 60 μg/dl indicated significant lead poisoning. In 1971 the threshold for blood lead levels was narrowed down to 40 μg/dl. This was eventually lessened to 30 μg/dl in 1975 and to 25 μg/dl in 1985 (Su et al. 2002). In 1991, the Centers for Disease Control and Prevention statement concerning lead poisoning in young children redefined elevated blood lead levels as those $\geq$10 μg/dl and advocated a new set of guidelines for treatment of lead levels $\geq$15 μg/dl (Roper et al. 1991).

Lead in Aquatic Environment

Water is the elementary requirement to sustain life on earth. Surface water receives several metals from both natural and anthropogenic sources. The concentration of metals in water as well as sediments depends on the kinds of rocks or soils present along the watershed. Due to the changing environmental conditions and extreme utilization of heavy metal-containing agrochemicals, these are being accumulated in soils which are then transferred to the water system by leaching.

The conclusive nemesis of Pb in the aquatic environment is shaped by the intrinsic chemical and physical properties of the water, like its acidity or alkalinity (pH), salinity, oxidation status, flow rate, suspended sediment, and dissolved or suspended inorganic and organic matter, i.e., hardness (Sepe et al. 2003). The pH of the water is significant in regulating the chemical fate of lead in terms of solubility, precipitation, or organic complexation. In water, Pb can prevail in four different forms – highly mobile and bioavailable (ionic form), in a bound form with limited mobility and bioavailability (organic complexes with dissolved humus materials), strongly bound with limited mobility (attached colloidal particles such as iron oxide), or very limited mobility and availability (attached to solid particles of clay or dead remains of organisms) (Sadiq and Alam 1997). The form of Pb along with its speciation varies in freshwater and saltwater. In freshwater Pb primarily occurs as the divalent cation (Pb^{2+}) under acidic conditions and forms lead carbonate ($PbCO_3$) and lead hydroxide [$Pb(OH)_2$] under alkaline

conditions. However, in seawater the influence of chloride concentration on lead speciation is much greater due to the salinity, and consequently the prime species are lead chlorides ($PbCl_3^- > PbCl_2 > PbCl^+$) and lead carbonate ($PbCO_3$) (Turner et al. 1981).

Fish is a food of excellent nutritional value and provides high-quality protein and a wide range of vitamins and minerals, including vitamins A and D, phosphorus, magnesium, selenium, iodine, and polyunsaturated fatty acids. They are put at the top of the aquatic food chain and may accumulate large amounts of some metals from water. In addition, fishes are the most effective bio-indicative factors in freshwater systems, for the estimation of trace metal pollution and risk potential of human consumption (Authman et al. 2015). Fish accumulate the pollutant from water and food chain both directly and indirectly. Hence, the disposal of Pb-containing pollutants in water has adverse effects on the fish health and other aquatic organisms of the food chain (Fig. 2). The toxic effects and mode of exposure are diverse, and a comprehensive analysis is thus needed to determine the toxic effects of Pb exposure on aquatic organisms and humankind.

Uptake and Bioaccumulation of Lead

Lead enters the body by ingestion through the intestines, through the lungs by inhalation, through the skin, or even by direct swallowing and ingestion (Markowitz 2000). Inorganic lead absorption occurs throughout the respiratory and gastrointestinal tracts. For adults with occupational exposure, the most predominant route for

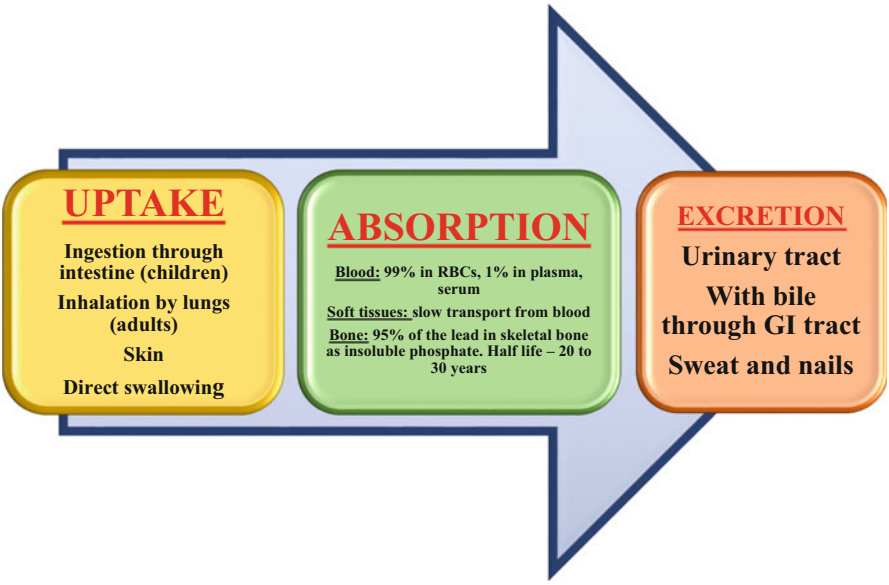

Fig. 2 Potential routes of lead exposure in aquatic organisms

absorption is through the respiratory tract. Rates of absorption via the GI tract depend on the nutritional status and the age of the individual exposed. Therefore, adults absorb an average of 10–15% of the ingested quantity, and this amount can increase up to 50% in infants, young children, and pregnant women. Absorption through the gut is the significant route for children. There is minute transcutaneous absorption of lead when inorganic lead compounds, such as those found in paint, are applied to the skin. On the contrary, organic (tetraethyl) lead, found in gasoline, can be absorbed through the skin (Papanikolaou et al. 2005).

The accumulation of heavy metals by aquatic organisms comprises of tissues that serve as the site for uptake and absorption like the gills, skin, and intestine. Gills have the largest surface area with respect to exposure and are in contact with water and thus are easily affected by the pollutants in the aquatic system. Hence, the gills are good indicators of aquatic pollution. Degenerative changes are reported in lead nitrate-treated fish in gill filaments and secondary lamellae (Paul et al. 2019). These tissues can concentrate metals and therefore show relatively high potentials for accumulation. Fish are exposed to metal through food in the GI tract and water in the gills; the liver detoxifies and releases metals that enter through the intestine (Lee et al. 2019). Moreover, the distribution of the metal accumulated in different organs can vary depending on the exposure route, the concentration and duration of exposure to contaminants, and the fish species (Rajeshkumar et al. 2017). Vinodhini and Narayanan (2008) demonstrated a higher accumulation of heavy metals including Pb in the liver tissue of common carp, *Cyprinus carpio*. The liver is considered as a critical organ regarding the homeostasis and excretion of metal ions. Accumulated Pb binds with steroids in the bile and forms the bile-metal (Pb) complexes which is reabsorbed by the intestinal wall or excreted through feces (Sures et al. 2003). Being primarily taken up by the gills of fish, waterborne Pb also contaminates the fish food and is absorbed via the intestines and then assembled in various internal tissues such as the liver and kidneys (Rogers et al. 2003). The accumulated metals stored in different organs can be transported to the liver via blood, and a certain amount of Pb accumulating in the liver can be re-excreted into the gut via bile. While some metals are excreted externally through the kidney and gill, accumulated metals are stored in tissues or are toxic to target organs (Kim and Kang 2017). Of the various metals, Pb is one of the most accumulative toxic and nonbiodegradable heavy metals owing to its properties to easily bind oxygen and sulfur atoms in proteins to form a stable complex (Verstraeten et al. 2008). Previous studies have observed substantial accumulations in various tissues of fish exposed to Pb. In juvenile Korean rockfish, *Sebastes schlegelii*, exposure to dietary Pb induced a significant accumulation in specific tissues such as the kidney, liver, spleen, intestine, and gill (Kim and Kang 2017).

Absorption of Lead

Following exposure to lead, the element is absorbed into and transported by the bloodstream and accumulates in three compartments: blood, soft tissues, and bone (Fig. 3). In blood, approximately 99% of lead is found in the erythrocytes and 1%

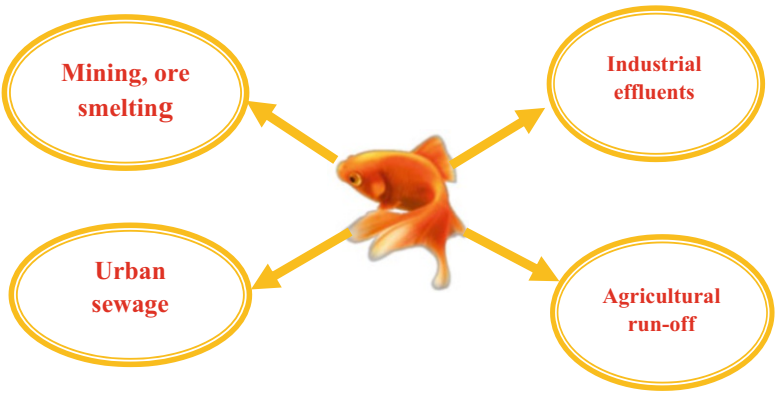

Fig. 3 Diagrammatic representation of lead metabolism

in the plasma and serum. The kinetics of lead transfer from blood to soft tissues is slow and takes approximately 4–6 weeks. Lead in blood has an estimated half-life of 35 days, in soft tissue 40 days, and in the bones 20–30 years. The initial distribution of lead throughout the body is dependent on blood flow to the tissues. More than 95% of lead is deposited in skeletal bone as insoluble phosphate. In adults, some 80–95% of the total body burden of lead is found in the skeleton, compared with about 73% in children. Lead concentrations in the bones and teeth increase as a function of age. Lead crosses the placental barrier, and concentration of lead in umbilical cord blood was found to be 80–100% of the maternal blood lead level. Several conditions known to increase bone turnover, such as pregnancy, lactation, chemotherapy, tumor infiltration of the bone, or postmenopausal osteoporosis, may be associated with the mobilization of lead in bone stores, leading to chronic lead toxicity.

Excretion of Lead

Inorganic lead is not metabolized. The hepatic P450 system oxidizes alkyl lead compounds. Generally, lead excretion is markedly slow, and the most important route is via the urinary tract. Lead may also be excreted with bile through the GI tract. Although minute amounts of lead are excreted through the sweat and the nails, these routes do not hold any practical significance. The biological half-life of Pb is estimated at 10 years, thus facilitating accumulation in the body. Studies by Philip and Gerson (1994) have shown that the use of chelating agents can elevate lead excretion in urine, and this constitutes the basis of the therapeutic approach to lead poisoning.

Mechanism of Lead Toxicity

It was shown that inflammatory, immunomodulatory, and oxidative stress mechanisms are responsible for lead-induced disorders, and these three mechanisms could interact and augment each other (Fig. 4).

- The inflammation process was speculated as the prime mechanism of lead toxicity inducing various disorders. Elevated inflammatory mediators have been also reported in lead-exposed animal and cell culture models (Sirivarasai et al. 2013). Lead poisoning can stimulate the inflammatory responses and tissue dysfunction which is linked with pathophysiologic conditions like asthma. The induction of inflammation and apoptosis by intracerebral implantation of a lead pellet in mammals can result in neuronal degeneration. Raised total WBC count was correlated with blood and urine lead concentration as well which affect liver and kidney functions (Patil et al. 2006).

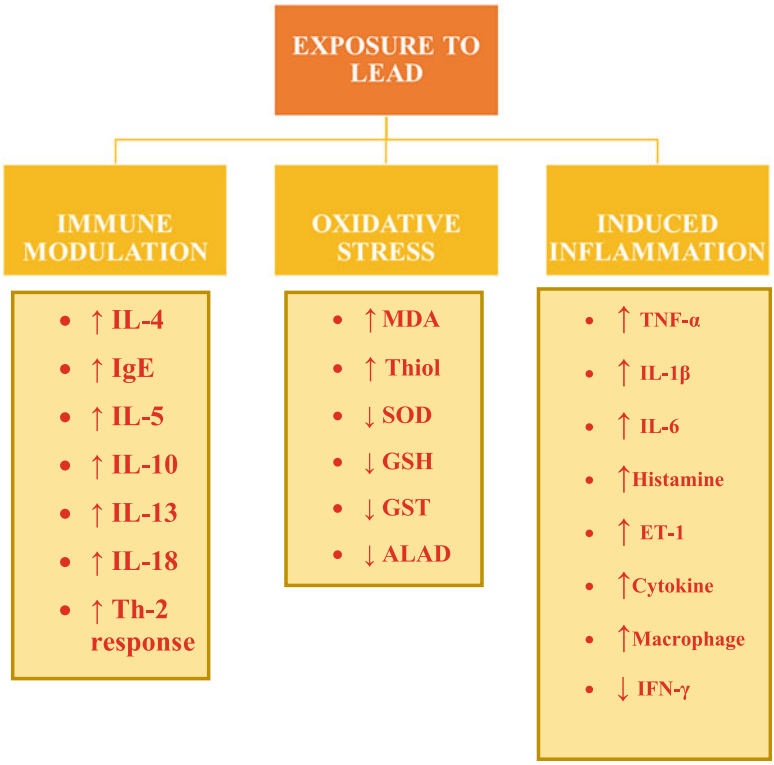

Fig. 4 Possible mechanisms of lead toxicity

- The other feasible means for lead-induced disorders is the immunomodulatory effect. Increased cytokines such as IgE, IL-1β, IL-4, IL-5, IL-6, IL-10, IL-13, IL-18, and IL-33 (Farkhondeh et al. 2017), circulating B lymphocytes, T helper subset development and function, increased TNFα level, and decreased IFN-ɣ level (Heo et al. 1996) were shown due to lead exposure. In human studies, increased eosinophil and the total immunoglobulin in asthmatic children with elevated BLL were reported which were related to higher asthma severity (Mohammed et al. 2015). Cytotoxicity effect, change in neurotransmitter storage and release, brain cell apoptosis, inflammation, and oxidative stress were proposed as the cardinal mechanisms of the neurotoxic effects of lead (Lidsky and Schneider 2003).
- Oxidative stress also imparts lead-induced toxicity (Fig. 5). Induction of oxidative stress (ROS production) in serum and the cell media was shown due to lead exposure through impairment of antioxidant activities of SOD, GSH, CAT, GPx, and increased MDA, H_2O_2 levels (Abdou and Hassan 2014). Increase of oxidant markers and decrease of GSH, SOD, and CAT were outlined in the nervous system of rat exposed to lead (Ghareeb et al. 2010). Decreased hepatic GPx, GST CAT, SOD, and GSH levels and increased MDA and H_2O_2 concentrations and elevated COX-2 gene expression were shown in rats exposed to PbA (Omobowale et al. 2014). Decreased NO via ROS-mediated NO inhibition in lead-exposed animals was reported which might lead to hypertension and tissue damage. Association of atherosclerosis and increased cardiovascular mortality with chronic occupational lead exposures and oxidative stress markers such as alteration in MDA and thiol levels were also reported. It was implied that oxidative stress (Vaziri et al. 2003) induces an alpha adrenoceptor-mediated vasoconstriction; impaired vasomotor tone because of reduced NO level, impaired NO homeostasis, and inflammation process are the possible mechanisms responsible for lead-induced hypertension and cardiovascular dysfunction. Oxidative stress was suggested as a major cause of lead-induced testicular damage (Makhlouf et al. 2008). The pathogenesis of lead toxicity depends on increased generation of free radicals including ROS, hydroperoxides, singlet oxygen, and hydrogen peroxide as well as decreased blood levels of GR, GPx, and GST in environmentally lead-exposed children and adults.

Effects of Lead Toxicity on Aquatic Organisms

Studies have evinced the possible inclusion of Pb in different physiological functions of fish and impairment of several cellular processes. The different doses at which lead induces toxic effects on some of the common fish species are listed under Table 2. Chronic Pb exposure causes iono-regulatory disruptions influencing Ca^{2+}, K^+, and Na^+ homeostasis in Fathead minnow, *Pimephales promelas* (Grosell et al. 2006). Iono-regulatory disruption during Pb exposure has been demonstrated in the rainbow trout, *Oncorhynchus mykiss* (Rogers et al. 2003). It has been exhibited in rainbow trout that the level of blood Ca^{2+} and Na^+ was constricted during exposure

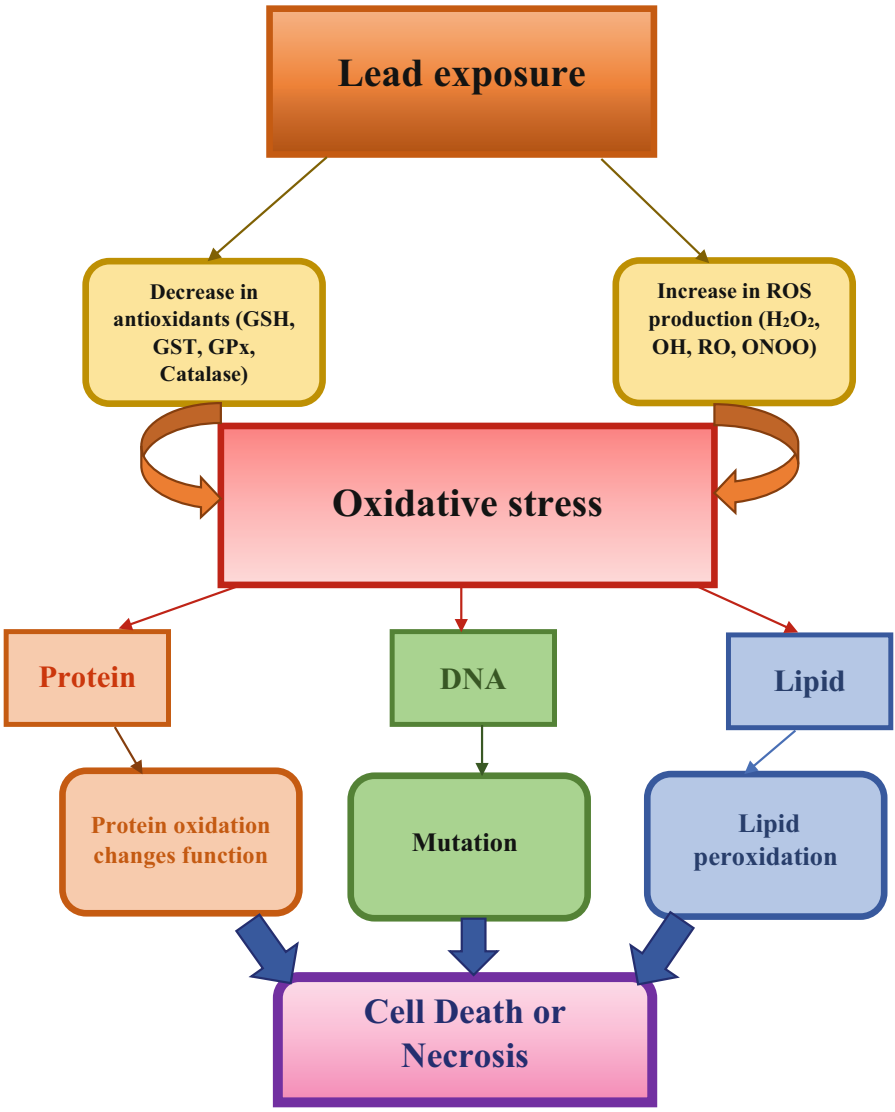

Fig. 5 Mechanism showing action of lead toxicity. Lead exposure leads to oxidative stress by hampering the redox homeostasis which causes damage to biomolecules ultimately resulting in necrosis

and the activity of Na^+/K^+ ATPase diminished significantly in a time-dependent manner. Alves and Wood (2006) reported high metal ion accumulation in the gills of the freshwater rainbow trout, *Oncorhynchus mykiss*, after exposure to waterborne Pb and subsequent inhibitory actions of Pb on Ca^{2+}, Na^+, and Cl^- uptake. Pb accumulation in different tissues led to elevation of reactive oxygen species (ROS) which

Table 2 LC_{50} values for different lead compounds on different fish species

Sl. No.	Common name	Scientific name	Lead compound	LC_{50} value (96 h)	References
1.	Rohu	*Labeo rohita*	Lead nitrate	34.20 mg/l	Singh and Manjeet 2015
2.	Common carp	*Cyprinus carpio*	Lead nitrate	77.33 mg/l	Abedi et al. 2012
3.	Mrigala	*Cirrhinus mrigala*	Lead chloride	40.54 mg/l	Kim et al. 2020
4.	Catla	*Catla catla*	Lead chloride	31.25 mg/l	Kim et al. 2020
5.	Nile tilapia	*Oreochromis niloticus*	Lead acetate	324.38 mg/l	Utami et al. 2018

Table 3 Different oxidants which are generated during lead-induced toxicity

Sl. No.	Oxidants	Radical/non-radical	Chemical formula
1.	Hydroxyl	Radical	OH
2.	Hydroperoxyl	Radical	HO_2
3.	Superoxide	Radical	O_2^-
4.	Alkoxyl	Radical	RO
5.	Hydrogen peroxide	Non-radical	H_2O_2
6.	Ozone	Non-radical	O_3
7.	Peroxynitrile	Non-radical	$ONOO^-$
8.	Hypochlorous acid	Non-radical	$HOCl^-$

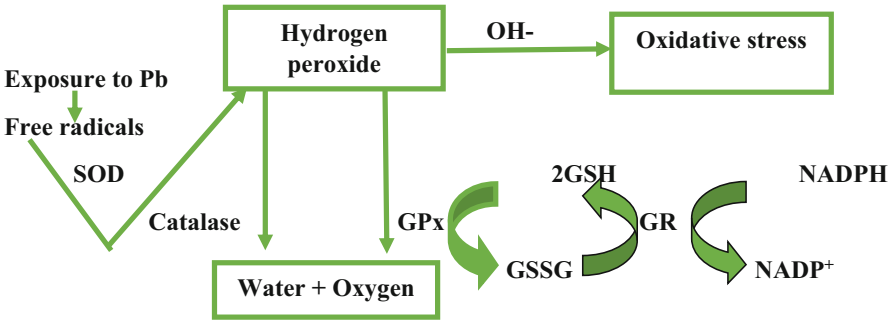

Fig. 6 Mechanism of oxidative stress in aquatic organisms when exposed to lead

causes redox imbalance and DNA damages in fish (Dai et al. 2012) (Table 3). The mechanism of lead-induced oxidative stress in fish is illustrated in Fig. 6. In many experimental studies, it has been observed that the activity of the antioxidant enzyme superoxide dismutase (SOD) was altered (increased or decreased) during exposure to Pb (Baysoy et al. 2012). Atli and Canli (2007) reported a significant augmentation in catalase (CAT) activity in the Nile tilapia, *Oreochromis niloticus*, after exposure to Pb. This may occur due to a defense mechanism to protect them from cell and tissue

damage by ROS generation. In contrast, Ates et al. (2008) have reported a considerable decrease in CAT activity in rainbow trout, exposed to Pb, which may be inhibited by binding metal ions to -SH groups on the enzyme molecule. Pb-induced oxidative modification in glutathione (GSH) metabolism along with the alteration in the activity of GSH-dependent enzymes has been reported in many fish species (Heier et al. 2009). Pb binds with thiol (-SH) groups, which is a cellular antioxidant or a component in several proteins and enzymes, such as GSH. Pb has a raised affinity for blocking thiol group functions and disrupts the oxidative balance (Hsu and Guo 2002). Oxidative stress by Pb toxicity induces δ-aminolevulinic acid dehydratase (ALAD) causing aminolevulinic acid (ALA) accumulation, and it also causes lipid peroxidation (LPO) in biological membranes (Antonio-García and Massó-Gonzalez 2008). The presence of several detoxication enzymes like 7-ethoxyresorufin-*O*-deethylase (EROD), 7-benzyloxy-4-trifluoromethyl-coumarin-*O*-debenzyloxylase (BFCOD), GST, and UDP-glucuronosyl transferase (UGT) was reported in the liver of goldfish because of the inhibitory effects of lead exposure at different doses (Boskabady et al. 2018).

There are so many evidences of Pb-induced histopathological changes in the gills, liver, kidneys, and spleen of different fish models. For example, Ramsdorf et al. (2009) reported that lead acetate induces micronucleus, chromosomal aberrations, DNA damages, and tailed nucleoids in the erythrocytes of fishes. Devi and Banerjee (2007) demonstrated that lead nitrate damages the histology of air-breathing organs of *Channa striata*. Lead acetate-induced micronucleus on cells of *Carassius auratus* was also reported by Çavaş (2008). Osman et al. (2010) established that lead acetate induces histopathological changes in the gills, liver, kidney, and spleen of *Oreochromis niloticus*. Studies of Mobarak and Sharaf (2011) revealed that lead acetate exposure resulted in various histopathological changes in the gills, liver, pancreas, stomach, and intestine of *Poecilia latipinna*. Ahmed et al. (2011) reported that lead chloride induces DNA damage using alkaline single-cell gel electrophoresis in the liver tissue of *Anabas testudineus*.

Any xenobiotic substance enters the body mainly through the intestine, so any damage to the intestinal lining can be a good indicator of toxicity. In *Channa punctatus* Pb causes severe damages in intestinal epithelium, disarrangement, and fragmentation of mucosal folding. Bacterial phagocytosis, intercellular killing capacity, and cell adhesion are also compromised with Pb toxicity. Release of antimicrobial substances like nitric oxide (NO) and myeloperoxidase (MPO) is also blocked. Chronic Pb exposure can lead to immunocompromisations like proliferation of lymphocytes (especially macrophages) and decreased TNF-α, suggesting both MAPK and NF-κB signaling, which in turn allows pathogens to gain access to the body (Paul et al. 2014).

Pb is a menacing neurotoxicant, resulting in neurodegenerative disorders, cell signaling deregulation, and neurotransmission impairment. Brain and cognitive function damages by morphological changes in the brain are also reported (Verstraeten et al. 2008). Pb exerts its neurotoxicity by impairing the calcium flux and calcium regulatory functions. Pb hampers neurotransmitter release and regulation by mimicking the calcium cation and binding to the calcium transport system of the nervous system. Therefore, Pb exposure induces changes in calcium

homeostasis, which affects neurotransmission (Westerink and Vijverberg 2002). Brain-specific proteins controlled by zinc finger proteins, the largest class of transcription factors, which allow binding to nucleic acids to regulate transcription are affected by Pb exposure due to the replacement of zinc by Pb ions. Pb toxicity also causes neurological injuries inducing severe hyperactive movements accompanied with hyperventilation in fish (Zizza et al. 2013). Richetti et al. (2011) have reported the inhibition of cholinesterase activity and resultant neurotoxicity in zebrafish induced by Pb. Exposure to Pb causes synaptic damage in fish, causing neurological and behavioral problems like loss of equilibrium, erratic swimming, and a high correlation between neurotransmitter systems and adenosine triphosphate (ATP). Pb exposure disrupts ectonucleotidase activity, a leading enzymatic pathway for the control of purinergic signaling. Thus, Pb exposure can cause structural or functional alterations in neuronal proteins, changes in gene expression, and disruptions in transduction and DNA repair processes (Richetti et al. 2011).

Pb causes immunotoxicity and modulates the immune responses of fishes causing their mortality (Small 2004). Decrease in the white blood cell and lymphocyte count in fish induced by cortisol secretion has been reported due to lead toxicity (Witeska 2005). Pb is known to affect the immune response by controlling the expression of cytokines in *Carassius auratus* (Yin et al. 2018). In crucian carp, it has been reported that mRNA expression of IL-10 and TNF-α was increased under Pb exposure and that certain Pb concentrations could have a serious damage on fish immune system (Dai et al. 2018). Many studies have shown that Pb has a negative impact on immune responses such as lymphocytes, leukocytes, inflammation, and apoptosis in fish.

In other studies, Pb is reported to be an endocrine disruptor in fish. The environmental Pb acts as a potent endocrine disruptor and affects ovarian steroidogenesis, gametogenesis, and ovulation of female Prussian carp (*Carassius gibelio*) (Łuszczek-Trojnar et al. 2014).

Therefore, studies have revealed the molecular mechanism of Pb-induced toxicity in fish from different experimental analyses. The multidimensional interaction of Pb ions with different biological macromolecules in an inhibitory way is the fundamental reason behind Pb toxicity. The effects of lead toxicity on various organs of different fish species are grouped in Table 4.

Impact of Lead Toxicity on Humankind

Lead is a poison that afflicts all the organ systems in the human body. Children are more susceptible to lead exposure than adults because of the more hand-to-mouth activity and an enhanced rate of intestinal absorption and retention (Shannon and Graef 1992). Organic lead is more toxic than inorganic lead as it is lipid soluble, which leads to rapid consequences (Timbrell 2008). However, the lead concentrations at which signs and symptoms appear vary widely and are dependent on the unknown characteristics of everyone (Bellinger 2008). Lead as an amassed toxicant affects cardiovascular, renal, neurological, skeletal, hematological, immune, respiratory, gastrointestinal, reproductive, and endocrine systems (Mitra et al. 2017)

Table 4 Effect of lead-induced toxicity on organs of different fish species

Fish	Source	Organs affected	Effects
Catla catla and *Labeo rohita*	Cauvery River, Karnataka	Gills, muscle, liver	Higher accumulation of Pb in *C. catla*
Cirrhinus mrigala	Ravi River, Pakistan	Gills, muscles, liver, kidneys	Biomagnification equating to positive correlation
Ctenopharyngodon idella	Lake Balaton and local fish farm, Hungary	Gut, gills, liver, muscles, and kidneys	Maximum concentration in kidneys and liver
Cyprinus carpio	Seyhan River, Turkey	Liver, gills, and muscles	High level of accumulation
Fin fish (*Oreochromis mossambicus*)	Jannapura Lake, Karnataka, India	Muscle and gill	Heavy metal toxicity due to anthropogenic activities
African catfish (*Clarias gariepinus*)	River Nile, Egypt	Liver	Enzymatic level and histopathological changes observed in liver tissues

Table 5 Effects of lead poisoning on different organ systems in children and adults

Sl. No.	Organ system	Children	Adults
1.	Renal system	Damage in proximal tubules Development of hypertension	Reduced glomerular filtration rate Hyperuricemia
2.	Cardiovascular system	High risk of blood pressure in later stages of life	High BP Atherosclerosis
3.	Nervous system	Behavioral problems Attention deficit Learning disabilities	Loss of memory Severe headaches Depression Numbness and irritation
4.	Digestive system	Abdominal pain Cramps	Loss of appetite Nausea Constipation
5.	Skeletal system	Bone growth affected	Reduced level of vitamin D leading to skeletal malformations
6.	Reproductive system	Not reported	Women – miscarriage, inhibited menstrual cycle and ovulation Men – reduced sex drive, sperm abnormalities

(Table 5). The most detrimental outcomes of lead are on erythropoiesis, kidney function, and the central nervous system (CNS) (Fig. 7). Propensity to baleful effects of lead also depends on the genetic makeup of a person. Many experimental along with epidemiological studies have associated gene-environment interactions and epigenetic regulations to have a plausible role in modification of the deleterious effects of lead in the human body. No matter what the route of exposure to lead is, the subsequent physiological effects are damaging.

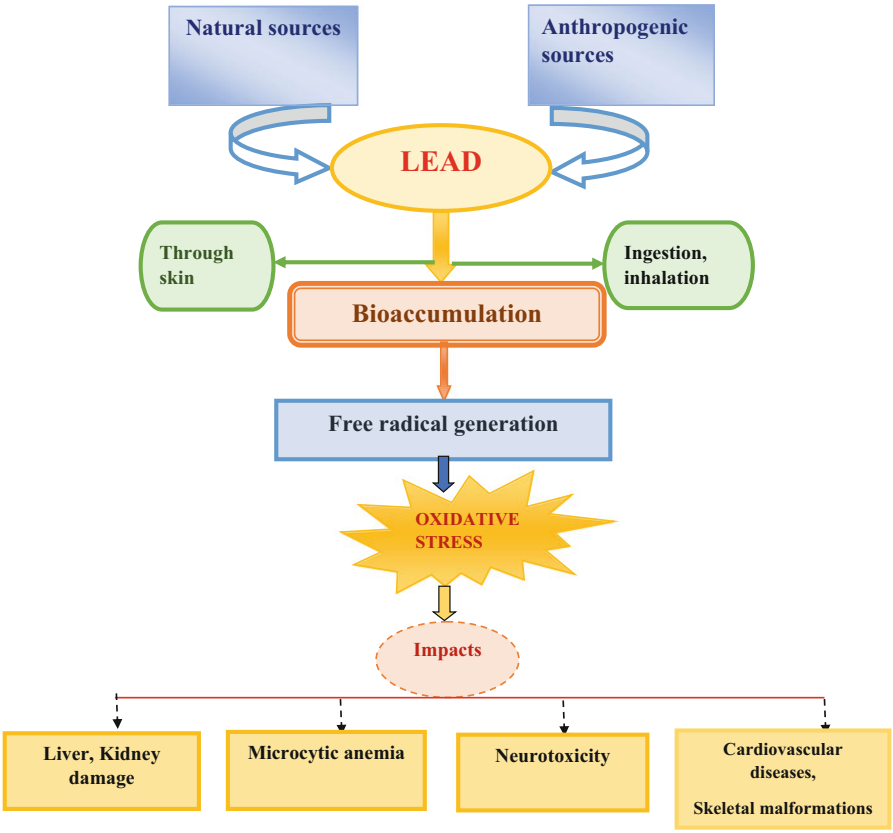

Fig. 7 Molecular basis of lead-induced toxicity in human organ systems

Hematopoietic System

The inimical effects of lead appear even with blood concentrations as low as 10 μg/dl. Lead poisoning affects heme synthesis, as lead inhibits the three main enzymes of the process, i.e., delta aminolevulinic acid dehydratase (ALAD), delta aminolevulinic acid synthase (ALAS), and ferrochelatase (Chisolm Jr et al. 1975). It is suggested that the inhibition of ALAD starts at concentrations as low as 5 μg/dl. As this enzyme is usually present in large quantities, the blockage of its activity might go discreet (Hernberg et al. 1970). Ferrochelatase catalyzes the incorporation of iron into the porphyrin ring. Due to lead toxicity, the enzyme is inhibited, or when ample iron is not present, zinc replaces iron, and zinc protoporphyrin concentration rises (Philip and Gerson 1994). The marked consequences of this effect are decrease in the levels of circulating hemoglobin and the inhibition of cytochrome P 450-dependent phase I metabolism. Lead toxicity leads to basophilic stippling of erythrocytes related to clustering of ribosomes and microcytosis when BLLs are 20 μg/dl. Consequently,

microcytic hypochromic anemia is frequently diagnosed in lead-exposed individuals. When compared with adults, children, especially those in their first year, develop certain harmful effects at lower BLLs, and lead-caused anemia has been related to age (Gulson et al. 2003).

Cardiovascular System

Lead toxicity has been related to soaring cardiovascular risk. But the impacts of Pb on the CVD system and blood vessels still need investigations (Navas-Acien et al. 2007). The threshold level below which lead may be reckoned innocuous is yet to be discerned. Many epidemiological and clinical studies have found a connection between chronic lead exposure and increased BP (Vaziri and Gonick 2008). Experimental studies have inferred a dose-response association between lead exposure and BP. Chronic lead exposure stimulates atherosclerosis in experimental animals, but the mechanism of lead-induced CVD risk remains unresolved. Defective antioxidant metabolism and oxidative stress might be hinted (Alissa and Ferns 2011). The other mechanism by which lead affects the CVD system is through the modulation of peripheral autonomic nervous system (ANS) ultimately resulting in chronic neuropathy (Zyśko et al. 2004). Acute and chronic lead toxicity causes morphological changes in the CVD tissues, including elevated apoptosis by activity of caspase-3, enhanced histone acetylation, and induced apoptosis in vascular and cardiac tissues (Xu et al. 2015).

Renal System

Renal toxicity due to lead poisoning entails inhibition in the proximal tubular lining cells and renal insufficiency. Acute lead poisoning leads to proximal tubular dysfunction which shows impairment in solute and amino acid transport resulting in Fanconi syndrome. Clinical features of Fanconi syndrome include aminoaciduria, glycosuria, phosphaturia, increased sodium, and decreased uric acid excretion (Loghman-Adham 1997). Chronic lead nephropathy includes gradual interstitial fibrosis, a decrease in the glomerular filtration rate, and azotemia (Orr and Bridges 2017). The acute form of nephropathy is most reported in children, and the chronic form is mainly reported in adults. Biomarkers of lead-induced renal injury include KIM1 and αGST (Yu et al. 2004).

Skeletal System

The skeleton functions as the primary reservoir of lead in the body as normal human adults can deposit 80–85% of available lead, while children can deposit 70% (Flora et al. 2012). It gets deposited in two regions – the exchangeable pool present at the bone surface and the non-exchangeable pool situated deeper in the cortical bone

(Kim et al. 2015). Lead intoxication impairs bone development, formation, and resorption and causes several pathological changes. The varied damaging effects of BLL as low as <10 mg/l include low levels of osteocalcin (bone growth protein) and impaired skeletal growth. This hampered bone growth is accompanied by unusual formation of bone organic matrix components. Vitamin D which plays a significant role in bone metabolism is reduced due to chronic lead exposure causing skeletal malformations (Kemp et al. 2007).

Nervous System

Lead toxicity affects the CNS with the brain being the most sensitive organ (Cleveland et al. 2008). Cognitive, affective, and physiological changes in the nervous systems are observed based on the dose and period of exposure. The mechanism accountable for neurotoxicity is not fully deciphered. Oxidative stress, reordered cell signaling, and neurotransmission are some of the important aspects reportedly involved in lead neurotoxicity. Lead poisoning results in free radical damage by generating reactive oxygen species (ROS) and the diminution of antioxidants. It binds to functional sulfhydryl groups of enzymes and makes them non-functional and contributes to an impaired oxidative balance (Reuben et al. 2017). Oxidative stress damages neurons by inducing the forkhead box class O transcription factor FOXO3, a main player in cellular pathways (Morris et al. 2015). Blood-brain barrier (BBB) sustains the brain integrity from toxic chemicals and metals (Bhowmik et al. 2015). Lead modulates calcium-dependent protein kinase C (PKC) pathway causing disruption of the BBB. It imitates Ca^{2+} and can cross the BBB swiftly, thus concentrating in the brain. Inside the brain, it solely interferes with neurotransmitter glutamate, which is pivotal for learning in the developing brain. The N-methyl-D-aspartate receptor (NMDA-r) is an ionotropic receptor for glutamate. Non-competitive antagonism of NMDA-r by lead in the hippocampus is one of the main mechanisms implied in neurotoxicity (Guilarte et al. 1994). So, all the calcium-dependent processes like calmodulin-II (CAM-II), neuronal nitric oxide synthase (n-NOS), and c-AMP response element-binding protein (CREB) are inhibited. Chronic lead toxicity causes neuropathy which includes weakness of distal muscles and marginal sensory involvement. Acute exposure to high lead concentrations results in motor neuropathy (Thomson and Parry 2006).

Respiratory System

Occupational lead exposure has been linked to impaired lung function (Khazdair et al. 2012). Several studies have reported permanent variations in respiratory system function as pulmonary function test values were highly reduced among lead-exposed workers as compared to the control subjects (Leem et al. 2015). Increased incidence of asthma in occupationally lead-exposed individuals has been reported through epidemiological studies (Dietert et al. 2004). BLL has been considerably related to

elevated bronchial responsiveness owing to raised IgE levels (Min et al. 2008). Experimental studies of lead poisoning on human lung epithelial cells have exhibited exposure of adenocarcinoma A549 cells to cytotoxic level of lead caused oxidative stress, as demonstrated by elevated ROS generation and lipid peroxidation, and decreased antioxidants like glutathione, superoxide dismutase, and glutathione peroxidase along with DNA damage (Lu et al. 2015).

Liver and Gastrointestinal Tract

The GI tract is the major route which is responsible for the intake and uptake of lead in the general population. Interactions between lead and nutrients like Ca and Fe affect lead bioavailability with liver being the largest soft tissue reservoir (Mushak 1991). Chronic abdominal pain, nausea, vomiting, constipation, bloating, and anorexia are the common GI symptoms seen with lead toxicity. Chronic lead toxicity causes a significant increase of liver enzymes ALP, ALT, AST, and GGT along with increased oxidative stress parameters (Mazumdar and Goswami 2014). Elevated lead concentration was linked to increased low-density lipoprotein cholesterol and decreased high-density lipoprotein cholesterol (Yang et al. 2017). Changes in basic processes including vitamin E and nitrogen metabolism, energy metabolism, oxidative stress, and the defense or detoxification mechanism have been observed owing to lead toxicity (Gao et al. 2017).

Reproductive System

Lead poisoning hampers both the male and female reproductive systems (Chen et al. 2016). During pregnancy, Pb crosses the placenta resulting in premature and intrauterine deaths and low birth weight. Different in vivo studies show that continuous exposure to lead might result in the inhibition of menstruation, ovulation, and follicular growth, delayed opening of the vagina, and a reduction in the frequency of implanted ova (Mendola et al. 2008). Usually, BLLs of >40 μg/dl are more detrimental to the human reproductive organs. Sometimes, even levels of <10 μg/dl Pb can be highly toxic (Balabanič et al. 2011).

The effects of lead poisoning on all the organ systems of the human body are elucidated in Fig. 8.

Applications to Prognosis and Other Diseases or Conditions

The molecular and biochemical bases of understanding lead-induced toxicity and its impacts on aquatic organisms and humans are elucidated. Toxicity of lead is responsible for redox imbalance and cytokine storm. Lead especially disturbs the inflammatory system and causes an increase in inflammatory mediators in humans. One of the major mechanisms underlying the toxic effects of lead on different organ

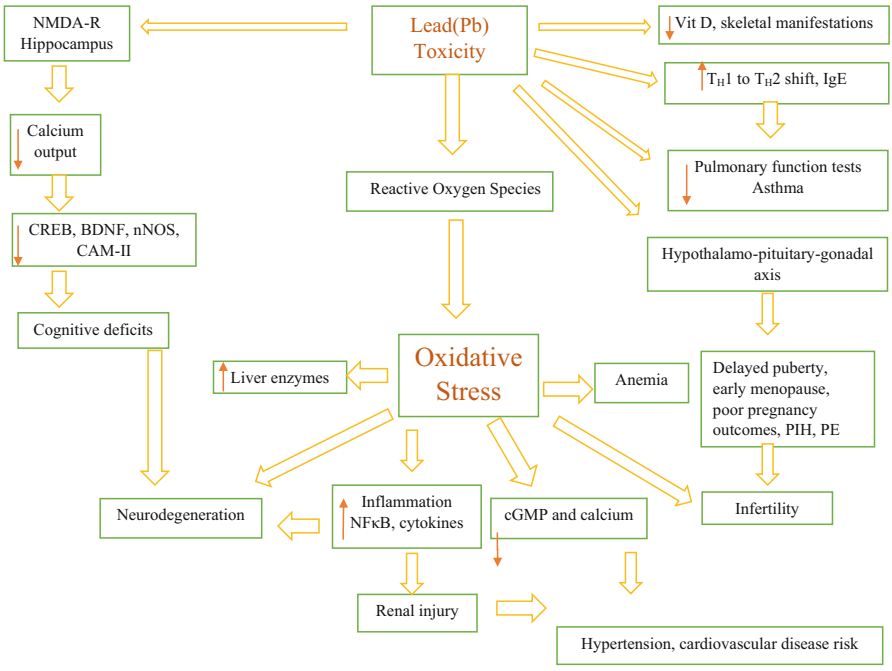

Fig. 8 Lead poisoning causes multiorgan manifestations

systems is inflammation. Like asthma, lead induces immunotoxicity by shifting the balance in T helper cell function toward T helper-2 subtype. Lead causes inflammatory cascade induction in CNS by activating glial cells and hampering the BBB function and increased expression of inflammatory mediators. Also, the role of oxidative stress in the induction of inflammatory responses should be considered. Lead disturbs the balance of oxidant-antioxidant system in the respiratory and nervous systems. A reduction in antioxidant content (GSH, SOD, and CAT) and increased MDA and NO in various tissues like liver and kidney after lead exposure are observed. Thus, the early detection and governing the levels of these biomarkers within the normal range may help to reduce lead toxicity and multiorgan impacts of lead poisoning, and it also may help to control the dilemma of uncontrolled environmental pollution by implicating strict actions (Malik et al. 2020).

Mini-dictionary of Terms

- **α-Glutathione S-transferase**: α-GST is a serologic marker of hepatocellular damage because of its low molecular weight, high cytosolic concentration, and short half-life. The main biological function of this enzyme is to detoxify the cells by conjugating glutathione with other electrophiles and hydroperoxides.

- **Catalase**: Catalase is an enzyme which is found in nearly all the living organisms which are exposed to oxygen. It decomposes hydrogen peroxide to oxygen and water. Additionally, it protects the cell from oxidative stress by generating reactive oxygen species.
- **Glutathione**: It is an antioxidant which prevents damage to cellular components caused by toxic metals, free radicals, and peroxides. It is made from the amino acids glycine, cysteine, and glutamic acid. The ratio of reduced glutathione to oxidized glutathione in a cell is a measure of oxidative stress where a high ratio indicates greater oxidative stress.
- **Kidney injury molecule 1**: KIM-1 is a type I membrane protein and is expressed in the kidney, liver, and spleen. It has different roles in immune diseases and kidney injury. It is also involved in HAV infections, autoimmunity, immune tolerance, and atopic diseases. KIM-1 is an early biomarker of acute kidney injury (AKI) and has a potential role in depicting long-term renal consequences.
- **Lipid peroxidation**: It refers to autoxidation process which is initiated by the attack of free radicals on phospholipids or PUFA of the cellular membranes or subcellular components, leading to the production of aldehydes, ketones, alkanes, carboxylic acids, and polymerization products. These products are highly reactive with other cellular components and serve as biomarkers of lipid peroxidation.
- **Malondialdehyde**: MDA is produced by the peroxidation of membrane PUFA. It is also generated during prostaglandin synthesis. MDA exists both as monomer and higher-order oligomers. Identification of MDA is used as a basic indicator of lipid peroxidation.
- **Superoxide dismutase**: SOD is the sole antioxidant enzyme that scavenges superoxide anion by changing this free radical to O_2 and H_20_2. Owing to this scavenging property, SOD has gained significant regard for therapeutic approach. SOD is widely used in anti-inflammatory, antitumor, radiation protection, and anti-senility applications.

Key Facts of Oxidative Stress

- It refers to the increase in the cellular concentration of oxidant or oxidizing agent as compared to the antioxidant levels.
- It is an imbalance between the formation and retention of oxygen reactive species (ROS) in cells and tissues and the capacity of a biological system to detoxify these reactive products.
- Superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen are generally known as ROS and are produced as metabolic by-products of cellular processes.
- When ROS production rises, they exhibit detrimental impacts on cellular biomolecules. Oxidative stress is accountable for the onset and/or development of diseases like cancer, diabetes, metabolic disorders, atherosclerosis, and CVDs.

- Lifestyle and dietary measures help in reducing oxidative stress in the body. This includes eating a healthy and balanced diet, regular exercising, minimizing stress, and reducing exposure to pollution.

Key Facts of Atherosclerosis

- It is the hardening and narrowing of heart arteries. It puts blood flow at a threat as the arteries are blocked.
- Symptoms of atherosclerosis include an unusual heartbeat, pain in the upper body, shortness of breath, paralysis, headache, high BP, and weakness in the legs.
- The disease starts with damage to the endothelium, and the common causes are high cholesterol and BP, obesity, inflammation, and unhealthy lifestyle.
- The complications of atherosclerosis include angina, kidney and heart diseases, stroke, heart attack or failure, and aneurysms.
- However, with a proper lifestyle and medications, the disease can be treated. Other treatment approaches include angiography and stenting, bypass surgery, endarterectomy, and fibrinolytic therapy.

Key Facts of Na^+/K^+ ATPase

- This is an enzyme present on the cell membrane of all animals.
- It is also known as Na^+/K^+ pump or sodium-potassium pump.
- The pump was discovered in 1957 by Danish scientist Jens Christian Skou. He was awarded a Nobel Prize for his work in 1997. The Na^+/K^+-ATPase functions to maintain resting potential, affects transport, and regulates cellular volume.
- It has a role as a signal transducer/integrator to regulate the MAPK pathway, reactive oxygen species (ROS), and intracellular $Ca2^+$.
- In the case of neurons, the pump is accountable for the maximum energy expenditure of cells.
- The pump moves sodium and potassium ions in opposite directions by utilizing ATP, each against its concentration gradient. In one cycle of the pump, three Na^+ ions are extruded from and two K^+ ions are imported into the cell.

Summary Points

- Lead is a broadly used metal, but it is concomitantly a multifaceted, subtle, and incessant poison.
- Fish are at the summit of the food chain in most aquatic environments and are thus the most vulnerable to the damaging effects of Pb exposure.
- Pb-induced toxicity in fish is predominately induced by bioaccumulation in specific tissues, and the retention mechanisms vary based on water habitat (freshwater or seawater) and pathway (waterborne or dietary exposure).

- Pb accumulation in fish tissues leads to oxidative stress owing to severe ROS generation and influences immune responses as an immunotoxicant.
- Fish are one of the most abundant vertebrates and can directly affect humans through food partake; hence, fish can be used to gauge the extent of environmental pollution in an aquatic environment.
- Pb induces a wide range of biochemical, physiological, and genetic malfunctions and as a collective toxicant afflicts almost all the major organ systems of human body.

Cross-References

► Biomarkers of Lead Exposure: Platforms and Analysis

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Per- and Polyfluoroalkyl Substances (PFAS) and Their Toxicology as Evidenced Through Disease and Biomarkers

44

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Abstract

Per- and polyfluoroalkyl substances (PFAS) are a group of at least 4730 chemicals with clearly important surfactant properties. They are used in coatings, paints, stain repellents, and firefighting foams as well as other industrial processes. Their high use and recalcitrance to degradation coupled with their lipophilicity and bioaccumulation makes them persistent organic pollutants or forever chemicals. In turn, they are measured in the serum of most humans, especially long-chain PFAS. The toxicity of most PFASs have not been studied, and the USEPA is starting a National PFAS-testing strategy to prioritize PFAS testing because testing all PFASs is not feasible. Some PFASs such as PFOA and PFOS are currently banned in many countries because of their persistence and bioaccumulation. Many PFASs have been observed in the environment and bioconcentrate in aquatic food webs. These chemicals are associated with immunotoxicity, cardiovascular disease, liver disease, reproductive and developmental disorders, and other human health issues.

Keywords

PFAS · Fluorosurfactant · Bioaccumulation · Persistence · Peroxisome proliferator activated receptors (PPAR) · Bioconcentration · PFOS · PFOA · Adverse outcome pathway (AOP) · Immunotoxicity · Nonalcoholic fatty liver disease (NAFLD) · Obesity · Development · Reproduction · Molecular initiating event

List of Abbreviations

6:2 FTS	1H, 1H, 2H, 2H-perfluorooctane sulfonic acid
ALT	Alanine aminotransferase
AhR	Aryl hydrocarbon receptor
AR	Androgen receptor
BCF	Bioconcentration factor
CPT1	Carnitine Palmitoyl transferase 1
CYP11A1	Cytochrome P450 11A1
CYP17A1	Cytochrome P450 17A1
ER	Estrogen Receptor
FABP	Fatty acid-binding protein
FASN	Fatty acid synthase
FOSA	Perfluorooctane sulfonamide
FXR	Farnesoid X receptor
GAC	Granular activated carbon
GR	Glucocorticoid receptor
HFPO-DA	Hexafluoropropylene oxide-dimer acid
HSD11B1	Hydroxysteroid dehydrogenase 11B1
HSD17B	Hydroxysteroid dehydrogenase 17B

HSD3B1	Hydroxysteroid dehydrogenase 3B1
IARC	International Agency for Research on Cancer
K_{oc}	Organic carbon-partitioning coefficient
K_{ow}	Octanol-water-partitioning coefficient
LDL	Low-density lipoprotein
logP	Partitioning coefficient
LPL	Lipoprotein lipase
LXR	Liver X receptor
MC4	Melanocortin 4
MetFOSA	N-methyl perfluoro-1-octanesulfonamide
NK	Natural killer
NRF2	Nuclear receptor erthyroid-2-related factor 2
OAT	Organic anion transporter
OATP	Organic anion transport protein
PFAS	Per- and polyfluoroalkyl substances
PFBA	Perfluorobutanoate
PFCA	Perfluorinated carboxylic acids
PFDA	Perfluorodecanoate
PFDoD	Perfluorododecanoic acid
PFHpA	Perfluoroheptanoic acid
PFHxA	Per-fluorohexanoic acid
PFHxS	Per-fluorohexanesulfonate
PFMOBA	Perfluoro-4-methoxybutanioc acid
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctanesulfonate
PFPeA	Perfluoropentanoic acid
PFSA	Perfluorinated sulfonic acids
PFTeDA	Per- fluorotetradecanoic acid
PFTrDA	Perfluorotridecanoic acid
PFUnDA	Perfluoroundecanoic acid
PGRMC1	Progesterone receptor membrane-associated component 1
POP	Persistent organic pollutant
PPAR	Peroxisome proliferator-activated receptor
PXR	Pregnane X receptor
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SCD1	Stearoyl coenzyme A desaturase 1
Srebf-1c	Sterol-regulatory element-binding factor 1c
STARD1	Steroidogenic acute regulatory protein
TAG	Triacylglycerol
THR	Thyroid hormone receptors
TNFα	Tumor necrose factor α
URAT1	Urate transporter 1

Key Facts of PFAS

There are approximately 5000 different PFAS chemicals produced.

PFAS chemicals, especially long-chain PFAS, have high octanol:water partition coefficients, low metabolism, and in turn long half-lives. Thus, they are persistent organic chemicals, sometimes referred to as "forever chemicals."

PFAS bioaccumulate in the food chain. They are often bioavailable in water and food, and therefore the primary route of exposure is dietary.

PFOA and PFOS, both with 8 carbons, are the most well-studied PFASs. They have long half-lives and are readily measured in the serum of most humans.

PFASs are associated with a number of adverse health outcomes including fatty liver disease, immunotoxicity, cardiovascular issues, thyroid problems, and reproductive and developmental disorders.

Introduction

Per- and Polyfluoroalkylsubstances (PFAS) are organofluorine chemicals used in stain repellents, varnishes, paints, cleaning products, semiconductors, firefighting foams, and other coatings. Fluorosurfactants are a subgroup of the PFAS that act as surfactants because of their fluorinated head and hydrophilic tail. There are at least 4730 different PFAS chemicals with more than three carbons. Currently, there are 100 s of PFAS chemicals in production in which there are little or no toxicity data.

The best biomarker of PFAS exposure is serum measurements. Many PFAS chemicals, especially long-chain PFAS, have long half-lives in part due to their recalcitrance to metabolism and adherence to serum and fatty acid-binding proteins. This is most likely due to the similar structure between PFAS and saturated fatty acids with the replacement of the hydrogens with fluorines. In turn, PFAS, especially long-chain PFAS bioaccumulate or bioconcentrate. Aquatic food webs and individuals that subsist on aquatic species are highly susceptible to this bioaccumulation. Humans are exposed primarily through their diet because of contamination in food and water. Persistence is the reason that PFOA and PFOS were banned.

Exposure to PFOS, PFOA, and other PFAS chemicals are associated with elevated markers of liver toxicity, perturbed lipid metabolism, higher serum cholesterol, and LDL. Much of these effects are associated with the PPARs that are activated by some PFAS in transactivation assays. However, there are discrepancies between studies in humans and rodents suggesting non-PPAR mechanisms or PPARγ antagonism. In addition to lipid metabolism and liver disease, PFAS are associated with immunotoxicity, including autoimmune disease and reduced reaction to vaccines. Reproductive problems and developmental delays occur due to occupational or environmental exposure. Hypothyroidism, cardiovascular disease, and kidney cancer are all associated with PFAS. These adverse effects will be further examined later in this chapter.

PFAS Types

PFAS can be amphoteric, nonionic, anionic, or cationic with the ionic or hydrophilic tailed chemicals primarily acting as surfactants. PFAS also vary in length, long or short-chain, and contain diverse functional groups such as carboxylic acids, sulfonic acids, sulfonamides, iodides, ethers, and phosphonic or phosphinic acids or may be made into polymers. The two major chemical forms or types of PFAS are sulfonates and carboxylic acids (Fig. 1); however, there are other PFAS types such as iodides, polymers, ethers, and phosphate-containing acids. Many of the ethers also contain sulfonate or carboxylate tails. PFAS are also characterized by the number of carbons that are present within the compound, with $\leq$6 carbons considered short chain and $\geq$7 carbons considered long chain. Characterization based on number of chains is especially helpful when considering potential environmental effects caused by bioaccumulation.

Some commonly used long chain fluorosurfactants such as PFOS and PFOA were phased out of production in the United States between 2002 and 2015 because of their long half-lives and subsequent bioaccumulation. All exemptions were phased out for PFOS in Europe in 2020 with only a few exemptions for PFOA remaining. In general, long-chain PFASs are now regulated in most of Europe under REACH (EC No. 1907/2006). However, PFOS and PFOA are still manufactured in China. Furthermore, short-chain PFAS chemicals are taking their place (Brendel et al. 2018). Because of their high production, variable association with soil, and bioaccumulation, several of the nearly 5000 PFAS chemicals are commonly found in food, groundwater, surface waters, and drinking waters. In turn, several different PFASs are regularly ingested by humans (Gleason et al. 2015; Baldwin et al. 2020).

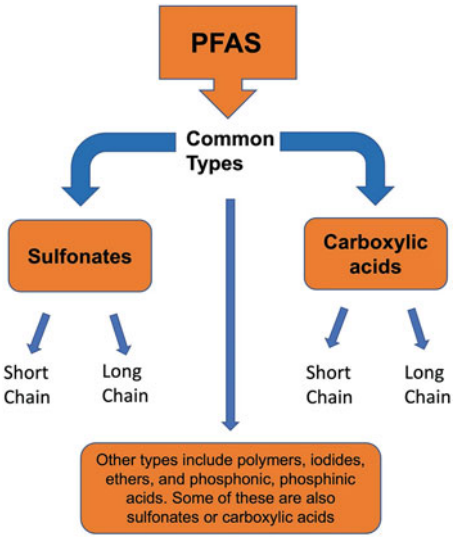

Fig. 1 PFAS chemicals come in many types. PFAS types include short chain, long chain, sulfonates, carboxylic acids, phosphonic and phosphinic acids, iodides, ethers, and polymers

Bioaccumulation

A primary concern of PFAS chemicals is their ability to bioconcentrate and bioaccumulate in the environment including aquatic ecosystems, food webs, and ultimately humans (Development 2006). Their lipophilicity, recalcitrance to degradation, and long half-lives, especially within the long-chain PFAS, are the primary drivers of their persistence. Therefore, PFAS are considered persistent organic pollutants (POPs) or sometimes known as forever chemicals (Baldwin et al. 2020).

Chemical and biochemical characteristics of long- and short-chain PFAS chemicals such as perfluorinated sulfonic acids (PFSAs) and perfluorinated carboxylic acids (PFCAs) contribute to their ability to absorb to substances such as water, soil, proteins, tissues, etc., and slow their elimination from the body. Equations used to describe the partitioning of chemicals within their environments include the octanol-water-partitioning coefficient (K_{ow}), which describes hydrophilicity and lipophilicity of a compound and can also be described using the partitioning coefficient (P), and the organic carbon-partitioning coefficient (K_{oc}), which describes the mobility of a compound in soil. Long-chain PFASs tend to have higher K_{ow}, log P, and K_{oc} values than short-chain PFAS (Fig. 2; Table 1). There is a strong correlation between the partitioning of chemicals in an aqueous environment and number of carbons (Fig. 2). Overall, the partitioning of chemicals within the environment contributes to their bioavailability to organisms. PFASs bioaccumulate and biomagnify along the aquatic food chain, and the bioaccumulation potential increases with an increase in fluorinated carbons in the chain (Martin et al. 2003b).

Body clearance is also effected by chain length. Short-chain PFASs are eliminated at higher rates in the urine than long-chain PFASs, primarily due to a poor affinity to renal reabsorption protein transporters such as the organic anion transport proteins (OATPs), and the organic anion transporters (OAT) by the short-chain PFAS. The main transporters associated with proximal tubular reabsorption of long chain PFAS, such as PFOA, include Oatp1a1, Oat4, and Urat1 and are typically involved in the influx of organic anions from the proximal tubules back into the kidneys, and this reuptake is less likely to occur for short-chain PFAS. Greater than 95–97% of PFOA is reabsorbed in mice depending on sex, and greater than 99.9% of

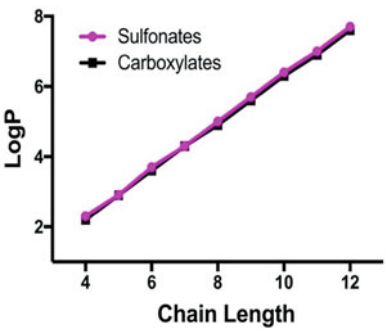

Fig. 2 There is a direct correlation between chain length and logP or octanol: water coefficients. The longer the chain length, the more lipophilic, the greater the bioaccumulation potential of these PFASs in animals. Sulfonates have slightly greater LogP values than carboxylates

Table 1 Log P and Koc values for long and short chain for select sulfonic and carboxylic acid-based PFAS

Sulfonates							
Long chain				**Short chain**			
Acronym	**Number of carbons**	**Koc**	**LogP**	**Acronym**	**Number of carbons**	**Koc**	**LogP**
PFDHpS	7	2.7	4.3	PFBS	4	1	2.3
PFOS	8	3	5	PFPeS	5	1.95	2.9
PFNS	9	3.73	5.7	PFHxS	6	1.78	3.7
PFDS	10	3.53	6.4	PFEtCHxS	8[a]	2.65	3.9[b]
PFUnS	11		7				
PFDoS	12		7.7				
Carboxylates							
Long chain				**Short chain**			
Acronym	**Number of carbons**	**Koc**	**LogP**	**Acronym**	**Length**	**Koc**	**LogP**
PFOA	7	2.35	4.9	PFBA	3	1.88	2.2
PFNA	8	2.39	5.6	PFPeA	4	1.37	2.9
PFDA	9	2.79	6.3	PFHxA	5	1.91	3.6
PFUnA	10	3.3	6.9	PFHpA	6	2.19	4.3
PFDoA	11	3.73	7.6	HFPO-DA	6[b]	1.92	3.6

Data primarily from (Kim et al. 2021)
aPFEtCHxS is composed of a carbon ring with a branch composed of two fluorinated carbons
bHFPO-DA is composed of two branches both containing three fluorinated carbons

PFOA is reabsorbed in humans (Han et al. 2012). For example, the total clearance of the short-chain PFAS, perfluorobutanoate (PFBA, C = 4), averaged 377.1 mL/day/kg while the total clearance of perfluorodecanoate (PFDA, C = 10) averaged 5.3 mL/day/kg in male rats (Han et al. 2012).

Sulfonates also accumulate more in tissues and have higher biomagnification potential compared to carboxylates; however, more evidence is needed when comparing depuration and accumulation of whole-body concentrations (Goeritz et al. 2013). LogP values are slightly greater in the sulfonates compared to the carboxylates by chain length (Table 1), but the differences are relatively small (Fig. 2).

Different PFASs are distributed to distinctly different tissues in humans with some showing preference for liver, lungs, or brain, and some with relatively equal distribution between kidney and liver, liver and brain, or liver and bone, although serum and skeletal muscle was not evaluated from the deceased individuals (Pérez et al. 2013). Feeding PFAS to rainbow trout did not cause significant biomagnification in comparison to bioconcentration through water exposures (Martin et al. 2003a). Waterborne exposures increased bioconcentration of each PFAS significant with bioconcentration factors (BCF) varying from 4 to 23,000. The highest concentrations of the different PFAS tested were consistently found in the liver with blood and kidney typically second and third, respectively. Skeletal muscle also accumulated different PFASs although concentrations were often 10X

less than liver. However, the bioaccumulation of each PFAS tested within muscle tissue indicates that humans are exposed through the aquatic food web (Goeritz et al. 2013). Fat is not considered a significant depot for PFASs despite their high logP values (Pérez et al. 2013), and instead the driving force for bioaccumulation is thought to be binding to albumin and serum and liver fatty acid-binding proteins (FABP) (Khazaee et al. 2021), many of which are abundant in the liver and serum and the cause of increased retention in these tissues. Increased kidney bioaccumulation was only observed in 8–9 carbon PFAS and may be caused by the reabsorption of long-chain PFAS through OATs and OATPs.

Environmental Distribution

There is also data that indicates K_{oc} increases with carbon chain length for all PFAS. The ability for certain compounds to adsorb to soil and sediments is tied to greater molecular mass and volume (Table 1). This contributes to greater retention of long-chain PFAS to the interfacial area of solids (Brusseau 2019). Adsorption is described as the adhesion of molecules to any surface. The adsorption of PFAS to solid-water, air-water, and oil-water surfaces are known to increase with carbon chain length. These hydrophobic interactions between molecules of greater mass and volume explain why longer-chain PFASs are found at lower concentration in water, especially groundwater, compared to that of short-chain PFASs as they are tied to the sediments or soils instead. Log K_{oc} also increases with a reduction in pH (Nguyen et al. 2020). With evidence that long-chain PFASs are more likely to be reabsorbed, bind to proteins in organ tissues, and adsorb to surfaces at greater rates, it is likely that long-chain PFASs will have higher biomagnification factors than short-chain PFASs (Fig. 3).

Environmental Problems

The accumulation of PFASs in waterways as a result of agriculture runoff and commercial production of surfactants has led to significant concentrations of PFAS in wildlife and humans. In Bahia, Brazil, a study looking at an estuarine tropical food web found concentrations of PFAS in tissues of bivalves, crustaceans, and fish species ranging from 0.01 to 1.20 ng/g of wet weight (wt) (Miranda et al. 2021). In bottlenose dolphins off the coast of South Carolina, USA, the average concentration of PFAS found in the blood serum was 2180 ng/ml in males and 1830 ng/ml in females (Fair et al. 2013). The respective concentrations for PFCAs in both sexes (221 ng/ml in males and 193 ng/ml in females) are significantly less than those of PFSAs (1700 ng/ml in males and 1420 ng/ml in females), once again indicating the increased retention and bioaccumulation of the sulfonates relative to the carboxylates. The accumulation of PFAS in wildlife can also transfer into people through

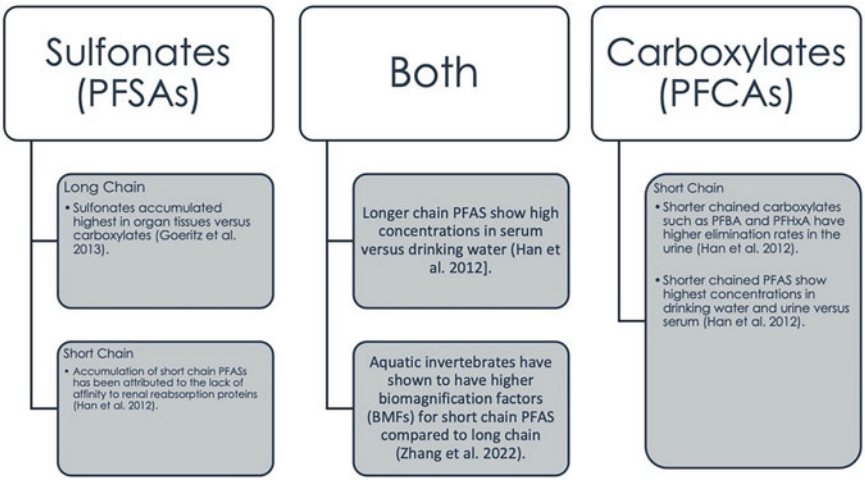

Fig. 3 Distinct characteristics between sulfonates and carboxylates along with differences between short and long chain

food and drinking water. In a study looking at the Gullah Geechee African American population in the Sea Islands off the east coast of the United States, the average concentration of PFOS, PFOA, PFHxS, PFNA, PFDA, and PFUnDA were 53.3, 6.0, 5.8, 2.3, 1.5, and 1.1 ng/g of wet weight, respectively (Gribble et al. 2015). The Gullah Geechee have a diet that is heavily reliant on seafood, and their consumption of contaminated fish has been posed as one of the main routes of exposure to PFAS. Overall, bioaccumulation in high-order species due to POPs such as PFAS continues to be a problem (Baldwin et al. 2020).

Some sources of drinking water pollution include aqueous film-forming foams (AFFF) that are frequently used near fire and military training facilities. In a study examining residential populations around Peterson Air Force Base (AFB) in Colorado exposed to PFAS-contaminated drinking water via AFFF, the average concentration of serum was PFCAs 3.93 ng/mL for females and 4.17 ng/mL in males from all three inspected residential areas while the average concentration of PFSAs was 24.4 ng/mL for females and 27.16 ng/mL in males (McDonough et al. 2021). A recent study out of an area of Sweden with a PFAS-contaminated municipal water supply due to firefighting foam use indicates that PFOS persistence can vary significantly depending on whether they are branched or linear. For example, branched PFOS such as 2/6 m-PFOS (2.67 years), 3/4/5 m-PFOS (3.43 years), and 1 m-PFOS (5.01 years) varied considerably between themselves and from linear PFOS (2.73 years). By comparison, PFOA has a half-life of approximately 2.47 years (Li et al. 2022).

Population studies on PFAS exposure via drinking water from polluted and unpolluted areas have shown variable concentrations of PFAS in human serum. In a study by the US National Health and Nutrition Examination Survey (NHANES)

looking at the general US population over 12 years of age, the average serum concentrations of PFAS are 20.7 μg/L (PFOS), 3.9 μg/L (PFOA), 1.9 μg/L (PFHxS), and 1.0 μg/L (PFNA) (Kato et al. 2011). In another population study looking at 69,030 participants of various age groups in Ohio and West Virginia, the geometric mean serum concentrations of PFOA, PFOS, PFHxS, and PFNA were 32.9, 19.2, 3.3, and 1.4 ng/mL, respectively (Frisbee et al. 2009).

PFOS and PFOA are the two most commonly studied PFAS, and both are long-chain (8-carbons) with different tails. PFOS is highly persistent in humans with a half-life of approximately 2.5–5.4 years (Olsen and Zobel 2007), and in turn it is present in most US citizens. For example, PFOS is detected in the blood of 98% of Americans with serum concentrations of approximately 1000 ng/mL in occupationally exposed personnel and 9–30 ng/mL in the general US population (Gleason et al. 2015). In summary, human exposure to PFAS is nearly ubiquitous, and this is demonstrated by the detection of the formerly high use of PFAS, PFOS in 98% of serum in US citizens. Serum concentrations vary widely between human populations depending on occupation, water contamination, and food source.

Remediation

Several methods of removing PFAS from soil and drinking water have been used with some proving to be more effective and others requiring more exploration. The use of granular activated carbon (GAC) with high adsorption capacity to trap PFAS molecules within micropores is an increasingly popular method. GAC has been used in the removal of legacy PFASs (PFOS and PFOA) in groundwater (Ochoa-Herrera and Sierra-Alvarez 2008). GAC is not as advantageous for removal of the new, short-chain PFAS that have less adsorptive qualities. Other forms of PFAS removal include the use of nanofiltration and reverse osmosis (RO). Nanofiltration and RO membranes showed 99% PFOS rejection efficiency for RO membrane and 90–99% rejection efficiency for nanofiltration membranes (Tang et al. 2007). *Pseudomonas* (bacteria) and *Phanaerochete* (fungus) are hypothesized to degrade fluorotelomers into shorter-chain PFASs such as perfluorobutanoate (PFBA) and perfluoropentanoic acid (PFPeA). Therefore, bacterial and fungal mediated degradation of PFAS has been tried recently; however, more research needs to be done in order to verify both methods as efficient means to reduce PFASs.

Wastewater treatment plants have adopted similar methods such as GAC or reverse osmosis for removing PFASs from drinking water; however, older wastewater treatment plants only use conventional methods that leave detectable traces of PFAS. Therefore, household filters should be considered as a method for PFAS removal. One study looked at point-of-use filters that reduced short-chain PFAS 72% and long chain PFAS 80%. These point-of-use filters contained coconut shell-activated carbon (CSAC) and a reverse osmosis (RO) membrane (Costello et al. 2022). Using mixtures of these different techniques has proven to be more efficient at PFAS removal than singular method types alone and may be useful in remediating the distinct types of PFAS (He et al. 2022; Tang et al. 2007).

Molecular Initiating Events

Molecular initiating events are the interactions between a molecule such as one of several PFAS and its unintended biochemical target such as a protein. There are several potential PFAS targets such as nuclear receptors, especially the peroxisome proliferator-activated receptors (PPARs), organic anion transporters (OAT) and other transport proteins, FABP, albumin, and uncoupling proteins. Several of these interactions have direct effects on the retention of PFAS such as binding to FABP, albumin, OAT, and OATP; others such as interactions with PPARs or mitochondrial uncoupling proteins effect energy utilization or development.

Nuclear Receptors

PFAS compounds, due to their variable lipid-like structures, act on a wide range of nuclear receptors and other transcription factors. This includes nuclear factor-erythroid factor 2-related factor 2 (NRF2) and nuclear receptors such as the PPAR, constitutive androstane receptor (CAR), pregnane X receptor (PXR), and estrogen receptors (ER) (Houck et al. 2021).

Twenty-four of the nuclear receptors in humans are ligand-dependent making them targets for disruption by xenobiotics. Similar to PFAS, several of these nuclear receptors are associated with metabolic changes such as obesity and metabolic disease, including the peroxisome proliferator-activated receptors (PPARs; PPARα/δ/γ), androgen receptor (AR), estrogen receptors (ER), glucocorticoid receptor (GR), constitutive androstane receptor (CAR), pregnane X-receptor (PXR), liver X receptor (LXR), farnesoid X receptor (FXR), and the thyroid hormone receptors (THRs) (Baldwin 2019).

ToxCast21 was perused for agonism and antagonism of several nuclear receptors by PFAS. Some of the nuclear receptors associated with obesity, in addition to the PPARs, were evaluated and shown in Table 2. Of the 5 nuclear receptors evaluated,

Table 2 Several nuclear receptors associated with obesity are activated by PFAS

Chemical	AR-*Inh*	PR-*Act*	PPARa-*A*	PPARa-*I*	PPARd-*A*	PPARd-*I*	PPARg-*A*	PPARg-*I*
HPDO-DA	X	X	2.1	X	X	X	X	X
PFHxS	X	X	11.2	X	X	X	27.5	X
PFNA	35.8	X	14.3	X	X	44.6	43.4	26.0
PFOS	X	X	11.2	X	32.2	X	20.3	X
PFOA	X	X	21.8	X	46.7	44.0	23.3	5.5-flag

Inh or I inhibition
Act or A activation
Flag inhibition was observed in 50% or less of replicates
X data not found: no effect or not tested
Data from ToxCast 21

CAR was activated by PFOA and PFOS; PXR was activated by PFHxS, PFOA, and PFOS; RXR was activated by PFHxS and PFNA; and LXR was activated by PFHxS, and ERα/β were activated by PFOS and PFNA and inhibited by PFNA.

Inhibition of the androgen receptor may cause obesity as androgens increase anabolism and the use of energetic macromolecules including the building of skeletal muscle. Testosterone also directly blocks adipocyte differentiation possibly by bypassing Wnt signaling. Antiandrogens also block androgen-mediated carnitine palmitoyltransferase (CPT1) transcription and in turn reduce the use of fatty acids in skeletal muscle leading to increased depuration into abdominal fat and obesity (Kim et al. 2019). Last, progestins are also potential obesogens. Progesterone increases the production of melanocortin 4 (MC4), a hormone that regulates appetite during pregnancy. This activity is enhanced by progesterone-mediated increases in mineralocorticoid receptor and leptin levels. In addition, progesterone can directly regulate adipogenesis through the progesterone receptor membrane-associated component 1 (PGRMC1), a membrane-bound progesterone receptor. Here, progesterone activation of PGRMC1 increases VLDL-R, LDL-R, and GLUT4 at the membrane directly increasing the uptake of fat and glucose into the adipocyte (including 3 T3-L1 cells) and therefore potentially increasing obesity (Furuhata et al. 2020).

Peroxisome Proliferator-Activated Receptors (PPARs)

Much of the research evaluating the molecular mechanisms of PFAS have involved PPARs, which are nuclear receptors responsible for the regulation of genes that contribute to lipid and glucose homeostasis, inflammatory responses, and fatty acid synthesis, storage, and mobilization. Several fatty acids are PPAR ligands, and fatty acids released by lipolysis during fasting trigger hepatic PPARα-mediated β-oxidation while inhibiting lipogenesis through the liver X receptor (LXR) (Yoshikawa et al. 2003). PPARγ and PPARδ are key in the uptake and use of fatty acids in white adipose and skeletal muscle, respectively.

A study that tested over 3000 PFAS chemicals in a concentration-dependent manner to determine receptor activation in comparison to reference agonists found that some PFAS activated ERα, NRF2, AhR, and RXRβ with ERα activated primarily by nonsulfonate / noncarboxylate PFAS. Very few PFASs activated LXR and FXR. The primary receptors activated were PPARs and PXR (Houck et al. 2021). PPARs were primarily activated by PFAS of various length with both sulfonates and carboxylates showing activity (Table 3).

PPARγ activation is associated with obesity most likely because of the increased depuration of fat from serum to adipose (Heindel and Blumberg 2019). In turn, several PPARγ activators such as thiazoldinediones, organotins (TBT), phthalates, and PFAS are associated with obesity and metabolic disease (Heindel and Blumberg 2019). However, PFOS induces wasting disease in rodents, the opposite of what PPARg activation causes. Recent research indicates that PFOS can inactivate PPARγ and in turn cause apoptosis in renal tubules (Wen et al. 2016). PFOS also perturbs

Table 3 Receptor activation by PFAS chemicals based on functional group. Several nuclear receptors are activated by PFAS chemicals of various types and sizes with PPARs showing the greatest number and diversity of activation

Receptor	Sulfonates	Carboxylates	Other
Estrogen receptor (ER)	Few (6–8)	Few (8–13)	Many (6–12) Iodide, phosphonic, acrylates, and alcohols
Nuclear factor-erythroid factor 2-related factor 2 (Nrf2)	Few (8–13)	Few (5–8)	Few (9–10)
Peroxisome proliferator-activated receptor alpha (PPARα)	Several (6–10)	Many (6–9)	Few (10)
Peroxisome proliferator-activated receptor gamma (PPARγ)	Many (6–10)	Many (6–10)	Few (9–10)
Aryl hydrocarbon receptor (AhR)	Few (4–10)	None	Few (3–10)
Pregnane X receptor (PXR) or PXR response element (PXR-RE)	Many (6–11)	Several (8–14)	Few (9–12)
Retinoic acid receptor β (RXRβ)	None	Many (8–12)	Few (6–10)

"Few" indicates 1 to 3 PFASs, "Several" indicates 4 to 7 PFASs, and "Many" indicates 8 or more PFASs. Range of number of carbons included in parenthesis
Derived from {Houck et al. 2021 #4303}

placental development through inhibiting PPARγ-mediated migration (Li et al. 2021). Thus, the roll of PPARg in PFOS-mediated toxicity needs more study.

PPARα activation should increase lipid metabolism and be antiobesogenic; however, there are some chemical PPARα activators associated with obesity. These chemicals are thought to primarily act during development to decrease androgen synthesis or reduce fetal lipid storage leading to undernourishment and a change in metabolic control that causes preferential storage later in life (Levin 2006). Overall, PPARγ activation is likely obesogenic and PPARα and PPARδ inhibition (antagonism) is likely obesogenic.

Short-chain PFAS are an emerging issue because they are being used in place of long-chain PFAS, and their effects are unknown. While they do not exhibit the same levels of cytotoxicity, short-chain PFASs do perturb similar pathways as long-chain PFAS. The PPARs are one of the receptor families perturbed by short-chain PFAS, particularly PPARγ (Houck et al. 2021). Short-chain PFASs have been shown to accumulate in adipose tissue (Liu et al. 2020) a major site of PPARγ expression, and this activation of PPARγ has been demonstrated to perturb adipogenesis markers such as SCD1, LPL, FASN, and others (Liu et al. 2020).

Molecular Biomarkers

Activation of PPARs and other transcription factors induce a variety of genes that can be used as molecular biomarkers of exposure and effect (Table 4). The diverse chemical structure of PFAS compounds has been of recent interest regarding

Table 4 Molecular biomarkers (transcriptional activation) of select PFAS chemicals

PFAS	CYP2B6[$]	NRIL3[γ]	NQO1	HMGCS1	SREBF1[αγ]	CD36[α]	FASN[γ]	CYP4A11[α]
HFPO-DA (6c)	25	25	25*	25*	nd	25*	nd	25
6:2 FTS (8s)	0.25	0.25	2.5*	0.25*	0.25**	0.25	2.5*	nd
FOSA (8s)	2.5	2.5	nd	nd	2.5*	nd	2.5	nd
metFOSA (9s)	0.25	0.25	2.5*	0.25*	0.25**	0.25	0.25*	nd
PFBA (4c)	0.25	0.25*	nd	0.25*	0.25**	nd	0.25*	0.25*
PFPeA (5c)	0.25	0.25	nd	2.5*	0.25*	nd	0.25	nd
PFHxA (6c)	0.25*	0.25**	nd	0.25*	0.25**	nd	0.25*	0.25*
PFHpA (7c)	0.25*	0.25**	nd	0.25*	0.25**	2.5	0.25**	0.25*
PFHxS (6s)	25**	25	25	25*	nd	25	nd	nd
PFOA (8c)	25**	nd	25*	25*	nd	25*	nd	25
PFOS (8s)	25**	nd	25*	nd	nd	25*	nd	nd
PFNA (9c)	nd	nd	nd	nd	nd	nd	nd	nd
PFDA (10c)	25**	nd	25*	25*	nd	25	nd	nd
PFUnDA (11c)	2.5	2.5	nd	nd	0.25*	nd	0.25*	2.5*
PFDoDA (12c)	2.5	0.25*	nd	0.25*	0.25**	2.5	0.25*	0.25*
PFTrDA (13c)	25	2.5	nd	nd	2.5	nd	nd	nd
PFTeDA (14c)	25	nd	nd	nd	nd	nd	nd	nd

() In parenthesis after the name of the PFAS is the number of carbons in the PFAS followed by whether it is a carboxylate (c) or sulfonate (s)
[$]Lowest concentration of three tested (0.25, 2.5, and 25 μM) in primary human hepatocytes that showed greater than 1.5-fold induction
*indicates statistical significance ($p < 0.05$) and ** indicates greater than twofold induction and statistically significant
nd not different
α PPARa reference agonist induced >2.0
γ PPARg reference agonist induced >2.0
Derived from {Marques et al. 2022 #4296}

molecular initiating events, as variations in structure may contribute to levels of toxicity depending on their biological activity. Parent PFAS compounds, such as the long-chain PFAS, PFOS, and PFOA, are more well researched due to their persistence in humans and the environment, while newer short-chain PFASs such as HPDo-DA and compounds derived from its breakdown have not been investigated until recently.

Marques et al. (Marques et al. 2022) recently published data which screened a variety of PFASs in primary human hepatocytes, including legacy, long-chain, and short-chain compounds, to determine changes in gene expression using 35 molecular biomarkers related to xenobiotic metabolism, oxidative stress, cholesterol synthesis, lipid synthesis, lipid transport, and lipid metabolism. This data showed that although many PFASs act on similar receptors and induce similar genes, short-chain PFAS are more potent inducers of changes in gene expression related to lipid synthesis and storage, xenobiotic metabolism, and cholesterol metabolism. They also observed short-chain PFASs increase lipid accumulation in these hepatocytes, which further suggests activation of PPARγ by short-chain PFAS.

PFAS activation of the PPARs and other receptors maybe, in part, responsible for pathology resulting from exposure. PFAS activation of PPARs alters liver lipid metabolism that may lead to steatosis. Many of the perturbed genes in these pathways have been associated with nonalcoholic fatty liver disease, including SREBF1, FASN, and CYP2B6 in humans (Hamilton et al. 2021). In addition to modulating the expression of genes that encode for lipid metabolism and transporters, murine *Cyp2b10* is highly induced in hepatocytes and mice exposed to PFOS (Rosen et al. 2010). PFOS moderately activates CAR and PXR in mice (Rosen et al. 2010) and induces murine Cyp2b9 and Cyp2b10 (Das et al. 2017).

We propose that the activation of PPARs may lead to a cascade that increases the sensitivity of several of the molecular biomarkers evaluated above (Fig. 4). PPARg activation likely induces SREBF1 and FASN. HMGCS1 is highly induced by FoxA2 (Howell and Stoffel 2009), which is primarily inhibited by insulin. Loss of insulin signaling during steatosis may increase FoxA2 activity. Interestingly, murine Cyp2b10 and human CYP2B6 are highly inducible by FoxA2 (Li et al. 2016). Given that CYP2B6 is also controlled transcriptionally by CAR and PXR (Hernandez et al. 2009), the activation of PPARs may lead to a cascade that allows for Cyp2b members to be sensitive biomarkers of PFAS.

Health Effects

As highlighted earlier, there are several fish, marine mammals (i.e. dolphin), and human populations with high exposures and serum concentrations of PFOS and other PFAS chemicals from environmental or occupational sources. Adverse health outcomes have been measured in multiple exposed populations with repressed immune responses and reduced responses to vaccines, increased cancer such as renal cell carcinoma, and reproductive and endocrine responses, including longer time to pregnancy, preeclampsia, and placental and milk transfer. Cardiovascular

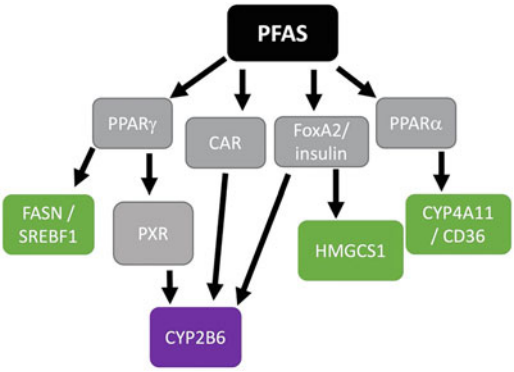

Fig. 4 Biomarkers of PFAS in liver cells. Several markers of energy utilization are perturbed by multiple PFASs; many of these are associated with activation of PPARs and other transcription factors. CYP2B6 is also a sensitive biomarker of PFAS exposure, potentially not only because it is induced by some key PFAS-associated transcription factors, but also because PPAR induces PXR, which may increase sensitivity. In addition, CYP2B6 is regulated by multiple transcription factors activated by PFAS

disease is increased, and this may or may not be caused by changes in metabolic disorders and liver disease associated with PFAS chemicals. The increased incidence and mechanisms associated with liver and metabolic disease may be the best understood of current PFAS-associated disorders as indicated by the molecular initiating events described above. Other adverse health effects include endocrine disruption, some of it mediated through nuclear receptors, increased oxidative stress, neurotoxicity, and pulmonary toxicity of which some may be elicited through reduced immune surveillance (Fig. 5). Greater detail is provided below.

Metabolic / Hepatotoxicity

PFAS cause hepatoxicity and the primary measured effect in most studies revolves around PFAS's ability to disrupt normal liver metabolic functions or increase fatty liver disease. PFOSs and other PFASs are known to cause wasting and increase hepatic steatosis in mice. PFOS also abrogates the beneficial effects of caloric restriction or metformin on fatty liver disease (Salter et al. 2021); therefore, exposure to PFOS may limit future treatment options which could be a serious problem given its long half-life. These effects may be caused by PPAR activation that regulates lipid metabolism, distribution, synthesis, and adipocyte maturation (Corton et al. 2014). The roles of PPARs in regulating several genes involved in fatty acid transport and synthesis are described above under molecular biomarkers and molecular initiating events. Steatosis is also associated with cardiovascular disease, and PFAS exposures are associated with progression of fatty liver disease to nonalcoholic steatohepatitis (NASH) in adults, children, and rodent studies (Jin et al. 2020).

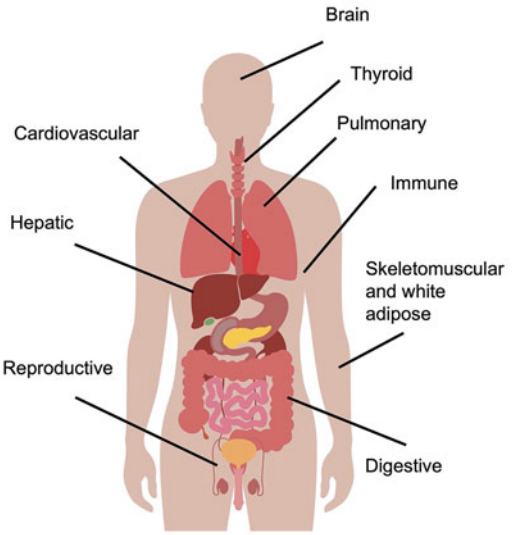

Fig. 5 PFASs have adverse health effects on several organ systems

A recent meta-analysis indicates that three PFAS, PFOS, PFOA, and PFNA, are associated with increased liver toxicity biomarkers in blood such as alanine aminotransferase (ALT) (Costello et al. 2022). Metabolic diseases occur in both males and females with females more sensitive to toxicity in mice and perturbations in glucose metabolism in humans, suggesting sexual dimorphism that leaves females more sensitive to PFAS exposure. Recent studies confirm sexual dirmorphism and indicate that PPARα is involved in the hepatoxicity and NAFLD (Sen et al. 2022; Hamilton et al. 2021), potentially due to early life exposures. However, it is also possible that PFOS and other PFAS induce steatosis by directly inhibiting mitochondrial β-oxidation and decreasing LDL content that, normally, would help transport triglycerides out of the liver.

The potent effects of PFAS on the liver are also likely caused by its hepatic bioaccumulation. OATs and OATPs increase absorption, and L-FABP binds and retains PFAS. For example, PFOS, PFOA, and PFNA have all been shown to bind FABPs with high affinity. Together, the enhanced FABP-mediated retention and greater nuclear receptor expression lead to greater hepatic PFAS concentrations and increased likelihood of activating (or inhibiting) PPARs.

Immune

There have been several epidemiological studies conducted on how the concentration of PFASs influences risk of autoimmune diseases. Most studies predominantly assess legacy PFAS, such as PFOA and PFOS; however, other PFAS (PFNA, PFHxS, and PFUnDA) were also assessed. Autoimmune and inflammatory diseases

associated with legacy PFAS exposure include Crohn's disease, osteoarthritis, rheumatoid arthritis, ulcerative colitis, and metabolic diseases such as diabetes. There are concerns that pulmonary diseases such as asthma are adversely effected by PFAS. For example, exposure of young mice to PFOS blunts airway antigens and inflammatory responses, including the major HDM allergen Der p1 at physiologically measured concentrations (nM) that ultimately perturb T-cell and other responses to pulmonary health. Ultimately, these inflammatory effects manifest themselves in several tissues and may be responsible for other organ system pathologies such as NAFLD, gastrointestinal disorders, cardiovascular disease, cancer, or pulmonary diseases (Wang et al. 2021).

Several studies have associated PFAS exposure with decreases in antibody concentrations and resistance to vaccines, and there is concern this also affects COVID vaccines. In a study of a birth cohort at the National Hospital in the Faroe Islands, prenatal exposure of PFAS was associated with a decrease in the presence of vaccine antibodies in children whose ages ranged from 5 to 7. Twofold increases in PFOA exposure resulted in drops of approximately 25% in serum antibody concentrations to tetanus and 39% in serum antibody concentrations to diphtheria (Grandjean et al. 2012). These effects have been confirmed in other populations of children (Timmermann et al. 2020). Adults may also suffer immunological effects as reduced white blood cell counts have also been observed in association with increased PFAS serum concentrations from an area in Ohio with contaminated drinking water (Lopez-Espinosa et al. 2021). Recent meta-analyses confirm these antivaccine effects of PFAS, especially PFOA and PFHxS, on the immune system for a couple of vaccines including tetanus, but with not enough data to confirm the effects for measles and several other vaccines. More time is necessary to determine and confirm the effects of PFAS on the Covid vaccines although early studies suggest repression of normal antibody responses and indicate PFAS exposure increases COVID-19 susceptibility.

In addition to reduced vaccine response, decreased response to disease has been observed in highly exposed populations. Exposure to PFAS is associated with poor Covid outcome, indicating an inability to amount a response. Research with B6 mice has demonstrated reduced natural killer (NK) and B-cell responses following exposure to several PFASs with the greatest effect observed following exposure to perfluoro-4-methoxybutanioc acid (PFMOBA). The mechanisms of these actions are not known but might be related to the immunotoxic properties of PPAR activation or regulation of reactive oxygen species, maybe through NRF2 signaling.

Developmental/Reproductive

PFAS exposure is associated with reduced pregnancies and a greater likelihood of miscarriage. The mechanisms are unknown, but the placenta may be a target. In a study from 1992 to 2002 by the Danish National Birth Cohort, women with greater than 26 ng/mL of PFOS and 3.9 ng/mL PFOA in their plasma were estimated to have higher odds of infertility and longer time-to-pregnancy (Fei et al. 2009). PFOA

antagonizes the expression of genes involved in oocyte maturation and embryonic implantation, which in turn is associated with later ages of menarche and irregular menstrual cycles.

PFAS are associated with lower testosterone levels and reduced sperm count in epidemiology studies. This work is further supported by rodent studies indicating adverse effects on adult and fetal testis. The mechanism of action is most likely due to reduced testosterone caused by increased aromatase activity leading to greater estradiol (increased aromatase may also cause cancer – see below) and/or decreased testosterone production within the Leydig cells of the testis. Several enzymes and steroid precursors required for androgen synthesis are repressed by PFOA or PFOS in Leydig cells including progesterone concentrations, STARD1, CYP11A1, HSD3B1, HSD17B, HSD11B1, and CYP17A1 (Tarapore and Ouyang 2021).

PFAS may also perturb the placenta through other possible mechanisms, including immunotoxicity related to NK cells. PFAS are associated with preelampsia, pregnancy-induced hypertension, gestational diabetes, and low birth weight. This occurs in humans and rodents, and studies suggest impaired transplacental support and disruption in placental angiogenesis while inhibiting placental PPARγ (Blake and Fenton 2020). Furthermore, PFAS can cross the placenta from the mother to the fetus or through milk from the mother to the infant with branched or linear forms showing different preferential transfer depending on the chain length. As PFAS concentrations increase in the infant, they decrease in the mother (Varsi et al. 2022).

Endocrine

PFAS bind a number of hormone receptors. For example, PFAS may disrupt the endocrine system, reproduction, and development through their activation or inactivation of the estrogen receptors, androgen receptors, or PPARs. PFAS also reduce endocrine action by inhibiting steroid synthesis as described above. There is some concern that observed metabolic or immune effects can be further exasperated by the displacement of cortisol or corticosterone from serum-binding proteins by PFAS due to its high affinity for fatty acid or lipid-binding proteins. Longer PFAS were more likely to bind these serum-binding proteins providing further support for a role in serum-binding proteins increasing the half-life of long-chain PFAS.

One of the greatest endocrine concerns associated with PFAS is hypothyroidism. A drop in serum thyroid hormone levels is associated with PFAS in pregnant females and infants (Lee and Choi 2017). In turn, lower thyroid hormones can effect several systems, especially during early development. This includes metabolism, reproduction, and development including neurodevelopment and mentation. PFAS may perturb thyroid hormone levels or action through multiple mechanisms, some of which have been studied best in Xenopus. These include perturbations in thyroid peroxidase activity or deiodinase activity, disruption of thyroid hormone receptors and/or feedback, competition for serum thyroid hormone-binding globulins by PFAS, impairment of iodine uptake, or crosstalk between PFAS-mediated PPAR activation and thyroid hormones (Coperchini et al. 2021). Further studies are

necessary to discern mechanism, but a combination of factors may play a role. Furthermore, thyroid hormone disruption may play a role in PFAS-mediated metabolic or development/neurodevelopmental disorders.

Cardiovascular

Excessive cases of arterial thrombosis have been identified in many studies investigating PFAS-exposed populations, suggesting a link between PFAS exposure and cardiovascular disease. The risk for cardiovascular disease in groups of individuals highly exposed to PFAS doubled compared to those not exposed or exposed to low levels. Peripheral arterial diseases were 75% higher in the highest quartile compared to the lowest quartile for PFAS exposure (Meneguzzi et al. 2021). PFAS are also associated with an increase in atherosclerosis-related vascular disease, hypertension in males, and recent research suggests PFOA exposure is associated with higher systolic and diastolic blood pressure. PFAS exposure is also associated with preeclampsia in pregnant women (Erinc et al. 2021). Modest links have been shown between high levels of serum PFOA and PFOS approximately five years after pregnancy with reported histories of preeclampsia. Adults and children exposed environmentally to high PFAS levels show increased total cholesterol, low-density lipoproteins (LDL), high-density lipoproteins (HDL), and decrease in triglycerides (Canova et al. 2021) causes of cardiovascular disease and highly regulated by the liver.

One possible mechanism for increased cardiovascular disease involves PFAS accumulation in platelets that leads to alterations in platelet function (De Toni et al. 2020). PFOA exposure is associated with increased membrane fluidity in platelets, as well as higher serum and platelet PFOA accumulation, increased activation at resting conditions, and increased calcium uptake and aggregation (De Toni et al. 2020). Increased reactive oxygen species may also play a role in greater cardiovascular risk as increased inflammation due to reactive oxygen species within the microvascular endothelial cells induced actin remodeling and membrane permeability (Qian et al. 2010).

Studies in model organisms support that PFAS induces cardiovascular toxicity. Zebrafish exposed to PFOA exhibited changes in cell signaling, muscle contraction, actin cytoskeleton, and gas transport with isolated heart cells being most sensitive (Yu et al. 2022). Maternal mice exposed to 20 mg/kg PFOA show mitochondrial damage in the hearts of the fetuses from exposed mothers with increased mitochondrial swelling, increased ROS formation, and decreased mitochondrial membrane potential compared to maternal mice that were not exposed to PFOA. However, this dose is much greater than those that induce NAFLD and hepatoxicity (Salimi et al. 2019).

Furthermore, some studies have shown no risk of cardiovascular in association with PFAS exposure. One study of farmers in nine rural districts in Sweden found no relationship between PFAS exposure and risk of cardiovascular disease (Mattsson

et al. 2015). A recent study in China also showed no association between PFOA exposure and hypertension (Zhang et al. 2022).

Neurotoxicity

Some laboratory-measured effects have been at doses significantly higher than those needed to elicit an effect on the liver or immune system; however, biochemical effects on GABA receptor have been at the low micromolar level (Tukker et al. 2020). The lower bioaccumulation in brain relative to liver or serum may greatly reduce the potential for neurotoxicity, however, increase blood-brain-barrier permeability in children, and during early development provides significant concern given the associations with reduced neurotransmitters, especially dopamine, which is key in movement, mood, and reward behaviors (Foguth et al. 2020). Adverse effects on neurobehaviors and hyperactivity have been observed, but further research is necessary as neurotoxicity data is lacking.

Cancer

PFOA has been classified as a possible human carcinogen by the International Agency for Research on Cancer (IARC), and emerging evidence suggests that PFAS chemicals may be associated with renal cell carcinoma based on occupational health-epidemiological studies. Furthermore, renal cell carcinoma patients have higher levels of serum PFOA concentrations (Shearer et al. 2020). The bioaccumulation and specific transport of PFAS chemicals across the tubules may play a role. Given that the newer, short-chain PFAS are transported across the kidney tubules with greater ease and bioaccumulate less, it will be interesting to see whether the association between PFAS and renal cell carcinoma decreases.

In addition, PFAS chemicals are associated with other cancers including testicular and to a lesser extent prostate cancer (Steenland and Winquist 2021). Carcinogenicity studies in rats confirm testicular cancer with significant increases in leydig cell cancers (Biegel et al. 2001). These cancers are thought to be induced through actions on liver PPARα that in turn increase estradiol levels. Rat carcinogenicity studies also suggest increased liver adenomas and pancreatic acinar carcinomas. Other cancers potentially linked but with either weak data or a need for more study include breast, non-Hodgkin's lymphoma, thyroid, and ovarian. Overall, more research is necessary to discern links and mechanisms between PFAS and cancer.

Summary

PFAS pose risk to environmental and human health regardless of chain length. However, long-chain PFASs pose special concern because of their persistence. PFAS are "forever" chemicals called persistent organic pollutants that build up in

the environment, and end up in surface and ground water and of course drinking water. They in turn bioaccumulate in many organisms from both biomagnification through the food web or bioconcentration through water. Humans also bioaccumulate PFAS, especially long-chain PFAS such as the highly used PFOA and PFOS, which are now discontinued in much of Australia, Europe, and the United States. PFAS are thought to primarily work through activation of PPARs and other receptors; however, PFAS also generate reactive oxygen species and bind several fatty acid-binding proteins.

Short-chain PFAS do not accumulate as greatly probably because they are less lipophilic and easier to eliminate through transport systems in the kidney. However, short-chain PFAS often show greater bioactivity on gene expression, most of which is probably initiated by specific nuclear receptors and other transcription factors. This greater activity thwarts some of the relief associated with the reduced bioaccumulation. As PFAS bioaccumulate, they become associated with organ system toxicity, especially liver/metabolic, reproductive, endocrine, and immunotoxicity. Remediation efforts to remove PFAS contaminants from water sources are being proposed and in some places utilized, but more research must be done to determine which method is most effective at removing PFAS of varying chain lengths and how to implement them on a larger scale to prevent further exposures within municipalities. An adverse outcome pathway is provided in Fig. 6. In

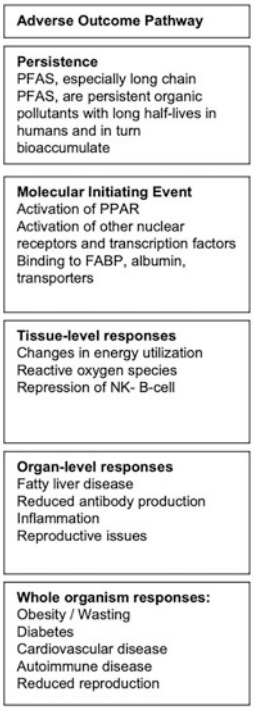

Fig. 6 Adverse outcome pathway for PFAS

summary, PFASs are persistent organic pollutants that perturb normal receptor activation, change energy utilization, and cause inflammation leading to a number of organ system pathologies that have been confirmed in both animal and epidemiology studies.

Mini-Dictionary of Terms

Adsorption:	The adhesion of a molecule to a surface.
Bioaccumulation:	An accumulation or increased concentration of a chemical substance with an organism.
Bioconcentration:	Increased concentration of a chemical substance in an organism relative to the media (typically water).
Biomagnification:	Increased concentration of a substance within the body from dietary sources.
Molecular initiating effect:	An initial chemical – macromolecule interaction that causes downstream effects.
NAFL:	Nonalcoholic fatty liver is a disease or condition in which fat, typically greater than 5% by weight, accumulates in the liver.
Octanol Water-Partitioning Coefficient (K_{ow}):	An equation used to describe the partitioning of chemicals to their environment; characterizes the hydrophilicity/lipophilicity of a compound.
Organic Carbon-Partitioning Coefficient (K_{oc}):	Equation used to describe the mobility of a chemical compound in soil.

Peroxisome Proliferator-Activated Receptor (PPAR):	PPARs are key nuclear receptors whose activity is regulated by fatty acids and fatty acid metabolites, and in turn they regulate a number of metabolic (energy utilization and storage), inflammatory, and developmental pathways.
PFAS: per- and polyfluoroalkylsubstances:	A large number of chemicals often saturated with fluorines that are used for coating purposes, firefighting foams.

Summary Points

- PFAS are common, and there are approximately 5000 different PFAS chemicals with the long-chain PFAS showing greater bioaccumulation.
- The bioaccumulation and general persistence of PFAS is of immediate concern. About 98% of US citizens have measurable serum PFAS.
- PFAS chemicals, especially long chain, have long half-lives and are poorly excreted leading to their bioaccumulation primarily not only through dietary exposure, but also through bioconcentration to fish from surface waters.
- PFOA and PFOS, both with 8 carbons, are the most well-studied PFAS. They have long half-lives and are readily measured in the serum of most humans.
- PFAS are cleared slowly because of binding to FABPs and poor excretion kinetics through transporters. Most PFASs are excreted through the kidneys.
- PFAS bind several receptors, and most of their adverse effects are thought to occur through modulation of PPARs.
- PFAS have significant effects on multiple organ systems with effects on energy metabolism, reproduction, inflammation, and immune status.
- Effects have been measured in most organ systems; the liver, immune, endocrine, and reproductive systems appear the most sensitive. This is confirmed by cell, animal, and epidemiology studies.

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The Complement System as a Biomarker of Environmental Pollutants as Toxic Agents: A Focus on Polybrominated Diphenyl Ethers (PBDEs) Exposure

45

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Contents

Abstract

Polybrominated diphenyl ethers (PBDEs) were the most widely used brominated flame retardants worldwide in recent decades. Due to their ubiquity in the environment and toxicity, PBDEs have posed great threat to both ecosystems and human health. PBDEs are commonly detected in aquatic ecosystems, causing

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bioaccumulation in fish tissues. In humans, food consumption (fish for example) and absorption of house dust are the major pathways of PBDEs exposure. Human and fish exposure to PBDEs are related to neurodevelopmental impairment, hepatotoxicity, metabolic, endocrine, and carcinogenic disruptions, as well immunological suppression. Among the components of the immune system, the complement system that plays a functional bridge between innate and adaptive immune responses has been evaluated after PBDE exposure in humans, mice, and fishes. Since its central role in immune response, the complement has risen as an important immunological indicator that may be used as biomarkers to monitor the effects of PDBEs exposure.

Keywords

Emerging pollutants · Persistent pollutants · Brominated flame retardants · ecosystems · Bioaccumulation · Human exposure · Complement system

Abbreviations

AChE	AcetylCholinesterase
BCR	B cell receptor
BDE	Brominated diphenyl ethers
BDE-153	2,2′,4,4′,5,5′-hexabromodiphenyl ether
BDE-209	3,3′,4,4′,5,5′,6,6′-decabromodiphenyl ether
BDE-47	2,2′,4,4′-tetrabromodiphenyl ether
BDE-99	2,2′,4,4′,5-pentabromodiphenyl ether
BFRs	Brominated flame retardants
C3(H_2O)	Thioester-hydrolyzed form of C3
C3a	Anaphylatoxin from C3
C3aR	Complement component 3a receptor
C3bBb	Second C3 convertase from Alternative Pathway
C3bBbC3b	C5 convertase from Alternative Pathway
$C3H_2OBb$	First C3 convertase from Alternative Pathway
C4b2b	C3 convertase from Classical and Lectin Pathways
C4b2bC3b	C5 convertase from Classical Pathway
C4BP	C4b binding protein
C5a	Anaphylatoxin from C5
C5aR1	Complement component 5a receptor 1
C5b-9	Terminal pathway complete complex
CR1	Complement receptors 1, CD35, C3b/C4b receptor
CR2	Complement receptors 2, CD21, C3d receptor
CR3	Complement receptors 3, CD11b/CD18 complex
FB	Factor B
FCDs	Follicular Dendritic Cells
FD	Factor D
FH	Factor H

FI	Factor I
GST	Glutathione S-transferase
HC	Hemolytic complement (C5)
LPO	Lipid peroxidation
MAC	Membrane Attack Complex
MASPs	Mannan-binding lectin-associated serine proteases
MBL	Mannose binding lectins
PBDEs	Polybrominated diphenyl ethers
POPs	Persistent organic pollutants
T4	Thyroxine

Introduction

Polybrominated Diphenyl Ethers Poisoning Around the World

Polybrominated diphenyl ethers (PBDEs) are listed as persistent organic pollutants (POPs) in the Stockholm Convention (Wu et al. 2020) and have been included among the substances of priority interest (Pardo et al. 2020). PBDEs are toxic, lipophilic, hydrophobic, and persistent artificial chemicals characterized by high physical and chemical stability (Wu et al. 2019). Due to these characteristics, PBDEs can bioaccumulate and biomagnify through the food chain (Montalbano et al. 2020). Even though their manufacture and use has been restricted, even banned, they are commonly applied as flame retardants in polymer products such as electronics, plastics, textiles, and building materials (Abdallah et al. 2015).

Large quantities of used and/or waste goods often flow from developed countries to developing countries (Odeyingbo et al. 2019). The current consumption of substantial quantities of PBDEs has been due to flexible regulations in the use and recycling of PBDE-containing materials and unawareness about PBDEs-mediated toxicities (Saini et al. 2019). By early 2000, the global production of commercial PBDE formulations was approximately 67,000 tons (Wu et al. 2011). According to Alaee et al. (2003), the US led North American demand for PBDEs representing approximately 50% of the total global demand and almost 95% of the most environmentally problematic and higher contamination is usually associated with densely populated urban areas.

However, in East Asian countries, including China, PBDE levels increased in the 1970s and there were no signs of a decline in this trend until recently. This increase, particularly in China, was due to the flourishing manufacturing industry, extensive legal and illegal import and recycling of PBDE-containing products from developed countries, and improper disposal and dismantling of e-waste (Yang et al. 2017). The routes of production and distribution of PBDEs in the environment, and human exposure are represented in Fig. 1.

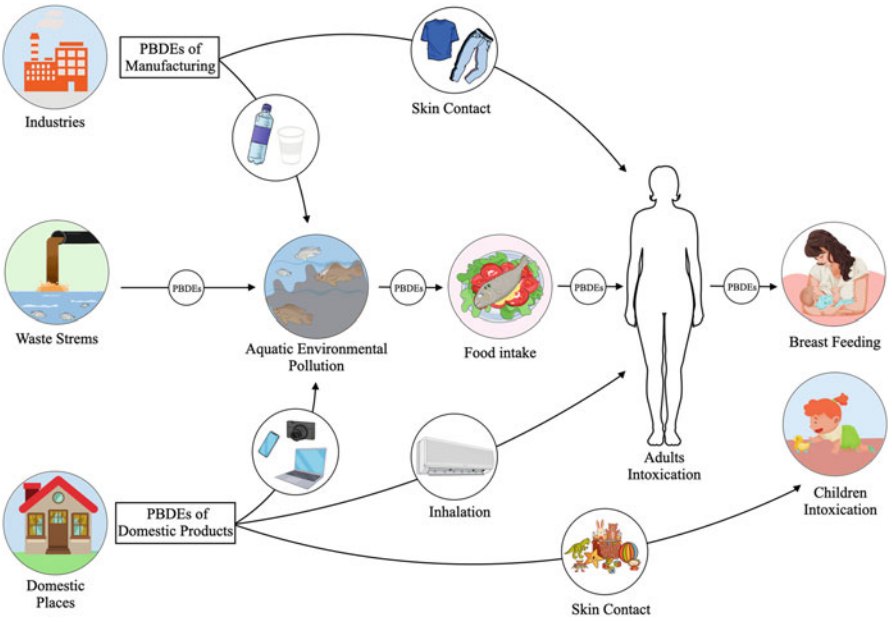

Fig. 1 Occurrence and exposure pathways of PBDEs. The diagram in this figure has been simplified to address the occurrence of these contaminants in the environment, as well as the forms of human exposure. The absorption of PBDEs in humans occurs in different ways, among their congeners. The oral route is identified as the most important, due to absorption through the diet, followed by the inhalation route and then the dermal route. Industries are responsible for the large production and release of products containing PBDEs into the environment, such as plastics, clothing, and electronics. The disposal of products containing plastics can reach water bodies, consequently the aquatic biota (various species of fish), and later humans, through water and/or trophic exposure. The clothes present direct contact with the man and consequently favor the dermal exposure. The industries are also responsible for the presence of PBDEs in ETE effluents, where the congeners remain in the final effluent, being later released to the receiving water bodies. Once released into the aquatic environment, PBDEs can also accumulate in plants and sediment. In confined environments, such as homes, PBDEs can be released from the polymeric mixture, enriching the dust that accumulates on household materials. Thus, when interacting with dust particles, PBDEs will accumulate in indoor particulate matter, being continuously deposited on surfaces and resuspended, and can be absorbed by inhalation and/or dermally. All these routes of exposure, whether by ingestion, inhalation, or dermic, can lead to the accumulation of these pollutants in humans. Children can receive PBDEs by mothers through breast milk and can be exposed due to longer residence times in indoor environments and hand-to-mouth habits, potentiating the problem for public health. (This figure was created using Canva Design: https://www.canva.com and Servier Medical Art, licensed under a Creative Commons Attribution 3.0 Unported License: https://smart.servier.com)

Polybrominated Diphenyl Ethers Group: Isomers and Congeners

PBDEs comprise of two halogenated aromatic rings bonded by an ester bond and are classified in relation to the number and position of bromine atoms in a particular molecule. Substitution of bromine atoms can take place at 10 possible positions on the two benzene rings resulting in 209 possible congeners (Rigét et al. 2006). PBDEs

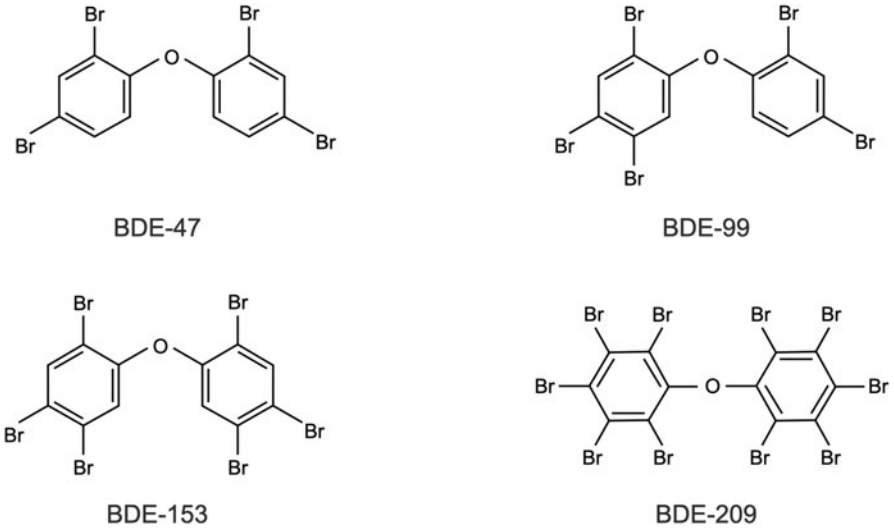

Fig. 2 Molecular structure of the most studied PBDE congeners. Molecular structure of BDE-47 (2,2′,4,4′-tetrabromodiphenyl ether), BDE-99 (2,2′,4,4′,5-pentabromodiphenyl ether), BDE-153 (2,2′,4,4′,5,5′-hexabromodiphenyl ether), and BDE-209 (3,3′,4,4′,5,5′,6,6′-decabromodiphenyl ether). BDE-99, BDE-153, and BDE-209 are examples of the penta-BDE, octa-BDE, and deca-BDE, respectively

were commercially produced in three technical mixtures, typically known as penta-BDE, octa-BDE, and deca-BDE, based on the number of bromine atoms (Wu et al. 2012). Penta-BDE is mostly used in polyurethane foams while octa-BDE is mainly used in rigid plastics and high-impact polystyrene. Deca-BDE formulation is a flame retardant used in a wide range of polymers including textiles, resins, and rigid plastics (Yogui and Sericano 2009). Laboratory tests have shown that the penta-BDE product is the most toxic among the three technical formulations, with deca-BDE exhibiting the lowest toxicity (Yogui and Sericano 2009). The deposition fluxes of PBDE congeners are significantly affected by diurnal and seasonal variability of meteorological factors and the temporal variation on PBDEs follow the order: summer > winter > autumn > spring (Niu et al. 2019). Molecules with one to four bromine atoms are classified as low molecular mass PBDEs, whereas the ones with five to ten bromine atoms are categorized as high molecular mass PBDEs.

Among the PBDE mainly studied congeners are BDE-47, BDE-99, BDE-153, and BDE-209 (Fig. 2).

Polybrominated Diphenyl Ethers in the Environmental and Human Health

PBDEs were first detected in the environment in the 1970s, and its environmental occurrence has been of increasing concern to scientists and policy makers since the 1990s (Andersson and Blomkvist 1981). These polymers are additives mixed into

but not chemically bound to the materials, so they can be released into the environment by leaching from the products. At present, PBDEs are recognized as a global pollution problem since they have reached remote areas such as the deep ocean, the Arctic and Antarctica (de Wit et al. 2006). They can be transported away from their sources for long-ranges through aqueous and/or terrestrial environmental compartments (Möller et al. 2011). PBDEs migrate to diverse environments throughout the production, use, recycling, and dismantling of PBDE-containing products (Hahladakis et al. 2018; Wu et al. 2019).

Many studies have verified the accumulation of PBDEs in natural environments (Darnerud et al. 2015; Wu et al. 2019), biota (Vorkamp et al. 2011; Xin et al. 2014), and even in human body (Abdallah and Harrad 2010, 2014; Stasinska et al. 2014) worldwide. Industrial plants manufacturing technical products along with facilities incorporating PBDEs into polymers are major point sources of these chemicals to the environment (Hale et al. 2003). Electronic waste recycling facilities were highlighted as point sources of PBDEs (Wang et al. 2005). Sewage treatment plants and landfills are also considered point sources since contaminants are concentrated in them from a variety of sources. High concentrations of PBDEs have been detected in both effluent and sludge from sewage treatment plants (North 2004). On the other hand, wear and tear of products containing PBDEs constitutes a diffuse, non-point source of these chemicals.

PBDEs tend to be stable and persistent in nature. These chemicals are often associated with soils and sediments. However, air and water particulate phases constitute important transport media for the dispersion of these contaminants. Many congeners can bioaccumulate in living organisms and biomagnify in food chains. Moreover, higher concentrations have been detected in aquatic biota as compared to terrestrial biota. Due to their high position in the food chain and the elevated exposure in the aquatic environment, freshwater fish often exhibit high residues of environmental contaminants (Leão-Buchir et al. 2021).

Limited data are available on the effects of PBDEs in man and wildlife (Niu et al. 2019). However, increasing scientific evidence has shown that several congeners have toxic properties (Eljarrat and Barceló 2018). According to (Darnerud 2003), effects of the penta-BDE formulation include neurobehavioral development disorders and thyroid hormone level alterations. Octa-BDE adverse effects include fetal toxicity and teratogenicity while deca-BDE negatively affects thyroid, liver, and kidney morphology. According to Dunnick et al. (2018) PBDE can induce transcriptomic changes and reduction of fertility in humans. Human health disorders like neurodevelopmental disability burden in the USA also strongly coincide with the introduction and use of PBDEs (Gaylord et al. 2020). PBDE residues have been detected in serum, breast milk, and adipose tissue from individuals (Meerts et al. 2002). In the case of children, hand-to-mouth activity and breast milk are two major exposure sources (Eljarrat and Barceló 2018). The main toxicological effects of PBDEs in the human body are summarized in Fig. 3.

Studies on effects of PBDEs in fish species have revealed changes in hematocrit and blood glucose as well as reduction in spawning success (de Wit et al. 2006). Recently, PBDEs have been proven to be endocrine disruptors affecting thyroid hormones in experimental systems (both in vitro and in vivo) (Darnerud 2008).

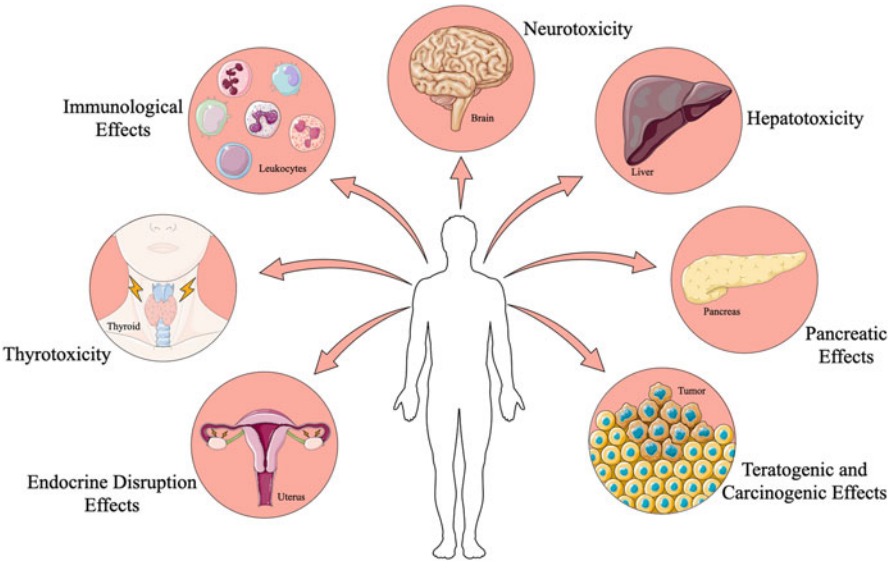

Fig. 3 Adverse health impacts of PBDEs. Of the various toxicological effects of PBDEs on the human body, the following have been identified as the most discussed: These compounds act as endocrine disruptors, altering the concentration of steroid, and thyroid hormone activities. PBDEs interact with the endocrine system by mimicking, antagonizing or altering the levels of endogenous steroids (androgens or estradiol). They interfere with neuroendocrine functions involved in embryonic development and reproductive potential. The effects of PBDEs on human thyroid function can interfere with the levels of triiodothyronine (T3) and influence TH levels. PBDEs can alter the intracellular oxidant/antioxidant balance and induce oxidative stress, mainly in the liver and brain. Exposure to PBDEs can cause disturbances in energy metabolism and induce higher levels of glucose and ATP, inducing diabetes. PBDEs also display neurotoxic effects, which can trigger inattentiveness, fine motor coordination, cognitive ability, hyperactivity and conduct problems, and motor skills. About the carcinogenic effects, PBDEs could covalently bind to DNA relying on the bromine substitution degree, causing DNA damage by inducing oxidative stress. PBDEs have been shown to be positively correlated with the risk of breast cancer in women and thyroid cancer. This shows a significant influence on PBDEs human exposure and human health risk. (This figure was created using Servier Medical Art, licensed under a Creative Commons Attribution 3.0 Unported License: https://smart.servier.com. Adaptations from the original art were made on pancreas and carcinogenesis)

According to Leão-Buchir et al. (2021) oral exposure to BDE-47 is toxic to *Oreochromis niloticus* as it caused decreased and increased levels of estradiol and T4, respectively, changes in AChE, GST, LPO, and histopathology, and led to impairment of antioxidant enzyme activity.

Polybrominated Diphenyl Ethers in Immune System

The immune system of teleost fish and mammals comprises innate and acquired immunity. Among the innate immune components are cells, such as macrophages, monocytes, granulocytes and natural killer cells, physical barrier, and complement

system, which plays a functional bridge between innate and acquired immune responses. The acquired immune system, in general, is composed of T- and B-cell subsets, which are responsible for the production of cytokines and antibodies, respectively (Bavia et al. 2022).

Freshwater fishes such as fathead minnows (*Pimephales promelas*) exposed to BDE-47 presented decreased pathogen resistance (Thornton et al. 2018). Likewise, anadromous fish species, such as Chinook salmon (*Oncorhynchus tshawytscha*), presented susceptibility to infection after exposure to BDE-47 and -99 (Arkoosh et al. 2015, 2018). The same was observed when fed with a diet containing five PBDE congeners (BDE-47, BDE-99, BDE-100, BDE-153, and BDE-154) (Arkoosh et al. 2010).

Regarding the effects of PBDEs on the immune system in marine mammals, cell line of harbor seals exposed to BDE-47, -99 and -153 had production of reactive oxygen species and phagocytosis affected (Frouin et al. 2010) while BDE-47, -100, and -209 exposure induced in an increase in inflammatory cytokine expression in dolphin's fibroblast cells (Rajput et al. 2018; Huang et al. 2020). In rats, BDE-209 exposure inhibits immune function suppressing lymphocyte proliferation, antibody production, and alters the structure of immune organs in rats (Liu et al. 2012). In humans, BDE-47 exposure has been associated to modulate inflammatory response in cell line (Longo et al. 2019, 2021), while BDE-47, -99, and -209 promoted oxidative stress, cell proliferation, and apoptosis dysregulation in human bronchial epithelial cells (Montalbano et al. 2020).

Complement System

In the last decade, the effects of PBDEs have been investigated on the complement system of humans, mice, and fishes (Kumar et al. 2014; Abrha and Suvorov 2018; Zhou et al. 2020). The evaluation of the complement system in mammals is an important immunological indicator due to its numerous functions in the defense of the organism. Mice and teleost fish have complement activation pathways similar to those of mammals. And complement proteins from fishes present many homologies to their mammalian counterparts. Likewise, functions such as opsonization, lytic activity, and modulation of the innate and adaptive immune response are also present in fish (Nakao et al. 2011; Bavia et al. 2022).

The complement system is an important component of innate and adaptive immunity, composed of more than 50 soluble and cell membrane-associated proteins. This system can be activated by three different pathways: Alternative Pathway, Classical Pathway, and Lectin Pathway (Fig. 4).

The activation of the Alternative Pathway is the result of spontaneous hydrolysis of the thiol-ester bond of protein C3, generating C3(H_2O), which can covalently bind on the surface of the pathogen. Factor B (FB) binds to C3(H_2O), which allows its cleavage by Factor D (FD), forming C3H_2OBb, the first Alternative Pathway C3 convertase. C3 convertase cleaves the component C3 in two fragments, C3a, a potent anaphylatoxin, and C3b. C3b is able to covalently bind to the pathogen membrane

and exposes a site to FB, which is cleaved by FD, which generates a second C3 convertase (C3bBb) of this pathway (Nilsson and Nilsson Ekdahl 2012; Bohlson et al. 2019).

The Classical Pathway is initiated when the C1q binds to immune complexes formed by antibodies of the IgG or IgM class. When C1q binds to antibodies the proteases C1r and C1s are activated. C1s cleaves C4 and C2 resulting in the formation of the Classical Pathways C3 convertase, C4b2b (Bohlson et al. 2019).

The Lectin Pathway is stimulated by carbohydrates, glycoproteins, and acetylated compounds present in abundance on the surface of bacteria, fungi, viruses, and parasites, which are recognized by collectins (mannose-binding lectins [MBL], collectin-10 [CL-10], and collectin-11 [CL-11]), and ficolins (1, 2 and 3). Once activated, the serine proteases associated with MBL (MASPs) cleave C4 and C2, forming C3 convertase, in the same way that occurs in the Classical Pathway (Garred et al. 2016).

All three pathways converge for the formation of C3 convertases, which cleaves the C3 protein into C3a and C3b. C3b then binds to this enzyme complex, forming the C5 convertase of each pathway, which cleaves C5 into C5a and C5b. This process results in the activation of the cascade of proteins of the common terminal pathway (C6, C7, C8, and C9), until the formation of the Membrane Attack Complex (MAC). MAC acts as a pore, allowing an osmotic imbalance, responsible for cell death (Merle et al. 2015).

Once activated, the complement system performs numerous functions against pathogens. Among the most important responses triggered by this system are: (1) stimulation of antibody production; (2) opsonization; (3) mediation of the inflammatory process; (4) cellular recruitment; (5) removal of apoptotic cells; (6) solubilization and elimination of immune complexes; and (7) cell lysis (Bavia et al. 2022). Some of these functions are presented in Fig. 4.

Effects of Polybrominated Diphenyl Ethers on the Complement System

Considering that POPs, especially BDE-47, BDE-99, and BDE-153, can alter metabolic activity (Arkoosh et al. 2017; Leão-Buchir et al. 2021) and the innate immune system (Arkoosh et al. 2010, 2015, 2018), we gathered here the main findings on the effects of these congeners on the activity of the complement System when different species of organisms were exposed to these pollutants (Table 1).

In order to evaluate the effects of POPs on human health, a cross-sectional study including elderly individuals (all aged 70 years) demonstrated that the exposition to POPs were associated with altered levels of complement system markers such as C3, C3a, C3a/C3, and C4. In particular, a negative association was found between the exposure to BDE-47 and the C3 plasma levels indicating activation of the complement system (Kumar et al. 2014).

In addition, another study that investigated the effects of BDEs exposure in follicular fluid, mural, and cumulus granulosa cells obtained from women undergoing in vitro fertilization showed that BDE-47 or BDE-153 was associated with

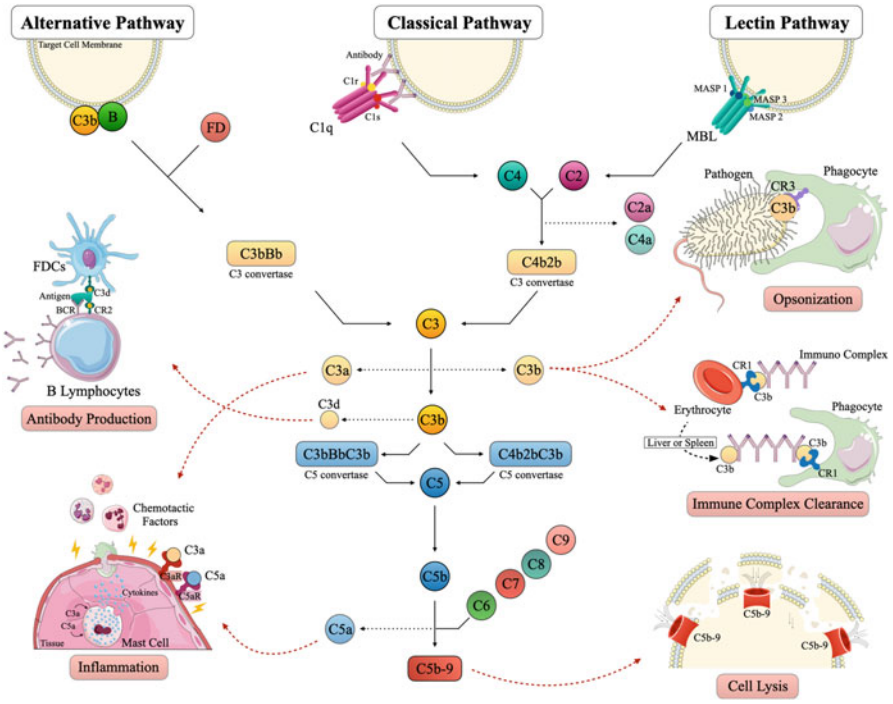

Fig. 4 Complement system activation and functions. Complement system is activated through three pathways: Alternative, Classical, and Lectin. **Alternative Pathway:** The spontaneous cleavage of C3 allows the protein to bind factor B (FB) and the target cell membrane. Thereafter, FB is cleaved by factor D (FD) into Ba and Bb, generating the C3 convertase (C3bBb) of this pathway, that cleaves C3 into C3a and C3b. **Classical Pathway:** C1q binds to IgG or IgM and activates C1r and C1s. C1s cleaves C4 into C4a and C4b and later C2 into C2a and C2b. C4b and C2b generate the classical pathway C3 convertase (C4bC2b). **Lectin Pathway:** MBL binds to the target cell membrane, resulting in the activation of MASPs. This activation leads to the cleavage of C4 and C2, which forms the Lectin Pathway C3 convertase (C4bC2b), similar to the Classical Pathway. Terminal Pathway: After the generation of C3 convertases from each pathway, C3 fragments are cleaved into C3a and C3b. C3b binds to C3 convertase and generates the C5 convertases (C3bBbC3b and C4bC2bC3b), which cleaves C5 into C5a and C5b. The C5b fragment binds to C6, and subsequently to C7, C8, and several C9 molecules, resulting in the formation of the Membrane Attack Complex (C5b-9n). **Complement Functions:** *Antibody Production*: The complement receptor 2 (CR2) of B Cells interact with C3d fragments bound to antigens. The antigen triggers B cell receptor (BCR), activates the cell, and increases the antibody production. The follicular dendritic cells (FDCs) are responsible for the antigen presentation that can also occur through CR2 and C3d. *Opsonization*: C3b and C4b bind to the target cell membrane which enables the interaction between the fragments and the Complement receptors (CR3) present on phagocytes. This interaction facilitates the pathogens phagocytosis. *Inflammation*: C3a and C5a act as chemoattractants to leukocytes, recruiting cells to the infected or damaged tissue. These fragments can also bind to the anaphylatoxin Complement receptors (C3aR and C5aR) expressed in endothelial cells and leukocytes, which increase the cytokine secretion and vasodilation. Immune Complex Clearance: The protein fragments C3b and C4b bind to immune complexes on the blood that are recognized by complement receptors (CR1) present on the surface of erythrocytes. This interaction allows the clearance of the immune complex to the liver or spleen and its elimination by phagocytes.

significant effects on gene expression in both cell types. Regarding the complement genes, *complement component 5a receptor 1* (*C5AR1*) gene was the third from 10 uniquely more expressed mural granulosa cell genes. Furthermore, an enrichment of complement genes was found in mural granulosa cells, being *C5* gene expression associated with BDE-47 exposure while *C3* and *FB* genes expression associated with BDE-153 exposure (Lefèvre et al. 2021). Since all these complement genes are involved with inflammation, and it is known that the triggering of innate immune pathways and inflammatory response are involved in ovulation (la Poulsen et al. 2019), it is suggested that a deregulated inflammatory response induced by BDE-49 and BDE-153 exposure may be associated with infertility.

Moreover, mice perinatal exposure to BDE-47 induced changes in the expression of genes involved in complement cascades in gonadal adipose tissue. The complement cascade genes such as *C9, C8A, C8B, C4BP*, *FI, HC* (or *C5*), *MBL1*, and *MBL2* were downregulated by more than 2-fold in mice exposed to BDE-47 (Abrha and Suvorov 2018). In addition, BDE-47 exposure affected the physiology of adipose tissue, and this tissue was proposed as a storage depot for the pollutant (Abrha and Suvorov 2018). Adipose tissue is one of the sources of complement proteins, and complement-mediated inflammation or dysregulation in lipid metabolism are associated with the pathogenesis of several metabolic diseases (Vlaicu et al. 2016).

Since BDE-47 is also found in marine environments, its effects on complement gene expression were investigated in the liver of marine fish medaka (*Oryzias melastigma*) (Ye et al. 2012). The expression of all six complement genes (*C1r/s*, *MBL2*, *Properdin*, *C3*, *C9*, and *F2* (coagulation pathway)) were downregulated in males after early exposure. On the other hand, in females, *MBL2*, *CFP*, and *F2* mRNAs expression were upregulated. In this way, the distinct gender difference in expression of six major complement system genes was evident in marine medaka under resting condition and dietary BDE-47 challenge. The authors suggested that the suppression of complement gene expression in BDE-47 exposed may be attributed to an increase of estrogenic effect induced by BDE-47 (Ye et al. 2012). Furthermore, the immunotoxic effects of BDE-47 was evaluated in rainbow trout (*Oncorhynchus mykiss*), also a marine fish. Serum level of C3, as well *C3* gene expression in head kidney, decreased significantly after BDE-47 exposure (Zhou et al. 2020).

Also, considering the aquatic environment, the effects of BDE-47 exposure were evaluated by high-throughput RNA sequencing in early development of zebrafish larvae (*Danio rerio*), a freshwater fish. Besides disrupting the eye and bone development of zebrafish larvae, BDE-47 exposure also downregulated complement gene expression from all pathways (*C1QA*, *C3*, *FB*, *FH*, *MBL2*, *C4B2*, *C8A*, *C8B*, *C9*, *C5AR1*, just to mention a few) (Xu et al. 2015).

Fig. 4 (continued) Cell Lysis: the formation of the Membrane Attack Complex (C5b-9n) results in cell lysis through osmotic imbalance. (This figure was created using Servier Medical Art, licensed under a Creative Commons Attribution 3.0 Unported License: https://smart.servier.com. Color and structure adaptations from the original art were made on cell membrane, tissue representation, phagocyte, mast cell, dendritic cell, lymphocytes, and bacterium)

Table 1 Effects of PBDEs on the complement system

Complement component	Organism	BDE	Tissue	Effect	Reference
C3, C3a, C3a/C3 and C4	Human	BDE-47	Plasma	Low C3 plasma levels	Kumar et al. (2014)
C5aR, C5, C3, and CFB	Human	BDE-47 and BDE-153	Follicular fluid, mural and cumulus granulosa cells	Upregulation in gene expression	Lefèvre et al. (2021)
C9, C8A, C8B, C4BP CFI, HC (or C5), MBL1, and MBl2	Mice	BDE-47	Gonadal adipose tissue	Downregulation in gene expression	Abrha and Suvorov (2018)
C1r/s, MBL-2, CFP, C3, and C9 (cell lysis), F2[a]	Marine fish *Oryzias melastigma* (male)	BDE-47	Liver	Downregulation in gene expression	Ye et al. (2012)
MBL-2, CFP, and F2	Marine fish *Oryzias melastigma* (female)	BDE-47	Liver	Upregulation in gene expression	Ye et al. (2012)
C3	Marine fish (*Oncorhynchus mykiss*)	BDE-47	Serum and head kidney	Decrease in serum level and downregulation in gene expression	Zhou et al. (2020)
C1QA, C3, CFB, CFH, MBL2, C4B2, C8A, C8B, C9, and C5AR1	Freshwater fish (*Danio rerio*)	BDE-47	Larvae	Downregulation in gene expression	Xu et al. (2015)

This table summarizes all studies regarding the modulation of the complement system after PBDEs exposure
[a]Coagulation pathway

Applications of Complement Components as Biomarkers of PBDEs Exposure

The identification of new immunological biomarkers for environmental biomonitoring adds to the biological studies already carried out within the area of ecotoxicology, expanding the range of analyzes and demonstrating in a more complete way the health status of organisms exposed to persistent pollutants. The

development of new biomarkers for monitoring the health of these animals will contribute to the elucidation of the toxicity mechanisms of PBDEs such as BDE-47, BDE-99, BDE-153, and BDE-209, and, consequently, opening perspectives for early intervention. In this chapter we propose the complement system as a new biomarker of PBDEs exposure which shows remarkable association with the extent of toxicity in humans, mice and fish. In particular, there was downregulation of complement genes and complement serum levels in humans and fishes after chronic exposure to BDE-47. Based on these findings, our hypothesis is that exposure to environmental pollution may exert an immunosuppressive effect on the complement system in humans, mice, and fishes, making them more susceptible to pathogenic infections. Thus, evaluating the effect of PBDEs congeners on the complement system shows that it is a strong candidate for biomarker of environmental toxic stress for different species.

Mini-Dictionary of Terms

- **Bioaccumulation**. Gradual accumulation of substances in an organism that occurs when an organism absorbs a substance at a rate faster than that at which the substance is lost or eliminated.
- **Biomagnify in food chains**. Increase in the concentration of a substance in an organism due to absorption from food and the environment.
- **Complement system**. Part of the immune system that is the first line of defense and enhances the ability of antibodies and phagocytic cells.
- **Estrogenic endocrine disruptors**. Are exogenous compounds, man-made chemicals that alter the functions of the endocrine system and cause various health defects.
- **Immune system**. Body's defense against infections and foreign substances.
- **Inflammation**. It is part of the complex biological process by which the immune system defends the body tissues from harmful agents, such as pathogens, damaged cells.
- **Persistent organic pollutants**. Organic compounds resistant to environmental degradation through chemical, biological, and photolytic processes.

Key Facts of Environmental Pollutants

- Polybrominated diphenyl ethers (PBDEs) are recognized as persistent organic pollutants (POPs).
- Polybrominated diphenyl ethers (PBDEs) are used as brominated flame retardants (BFRs).
- The slow elimination of PBDEs due to the environmental persistence has led to worldwide contamination of PBDEs.

- Human toxicity data demonstrated that PBDEs have extensively endocrine disruption effects, developmental effects, and carcinogenic effects among different populations.
- Humans can be exposed to PBDEs through inhalation of contaminated air, oral intake of contaminated food and/or dust, and dermal contact with dust and/or soil.
- The complement system is an important component of innate and adaptive immunity and may be a biomarker of environmental pollutants exposure.
- The complement system genes are downregulated after BDE-47 exposure in humans, mice, and fishes.
- Serum level of complement component C3 decreases after BDE-47 exposure in humans and fishes.

Summary Points

- PBDE compounds are organobromine compounds that are used as flame retardants.
- Humans and ecosystems are exposed to PBDEs through many activities.
- Concentrations of PBDEs in human and animal bodies are increasing.
- PBDEs may exert different toxic effects on different types of cells and tissues.
- BDE-47 exposure modulates complement gene expression in humans, mice, and fishes.
- Complement system is a promising candidate as a biomarker of PBDEs exposure.

Cross-References

► Biomarkers of Liver Injury due to Toxic Agents: Progress, Current Applications, and Emerging Directions
► Lead and Aquatic Ecosystems, Biomarkers, and Implications for Humankind

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Biomarkers of the Toxic Effects of Chemotherapeutic Agents: A Focus on Antimalarials

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Contents

Abstract

Malaria continues to inflict unacceptably high levels of sickness and mortality, as reported in the previous editions of the World Malaria Report. Antimalarial drugs are the most common treatment for this condition. The gastrointestinal tract, the eyes, the central nervous system, and the cardiovascular system are all affected by

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antimalarial toxicity; this literature aims to provide an overview of recent advances relating to biomarkers of the toxic effects of antimalarial drugs. Gene polymorphism of metabolizing enzymes; predominantly Cytochrome P450 (CYP) and UDP Glucuronosyltransferase (UGT), drug Transporter; (P-glycoprotein and Organic anion Transporter 1B1), Glutathione S-transferases and deficiency of Glucose-6-Phosphate dehydrogenase (G6PD) are seen as biomarkers of antimalarial drug toxicity. Individual differences in drug response are linked to polymorphisms in the genes encoding these markers. Despite multiple studies on the potential of biomarkers in antimalarial medications, further study is needed to better characterize biomarkers for specific adverse effects (AEs) associated with the use of antimalarial drugs.

Keywords

Malaria · Antimalaria · Chloroquine · Artemisinin-based combination · Metabolism · Toxicity · Resistance · Cytochrome · Drug transporter · Polymorphism · Prophylaxis

Abbreviations

ACTs	Artemisinin-based combination therapies
AEs	Adverse effects
AL	Artemether–Lumefantrine
AS+AQ	Artesunate + Amodiaquine
AS+MQ	Artesunate + Mefloquine
AS+SP	Artesunate + Sulfadoxine–Pyrimethamine
BCRP	Breast Cancer Resistance Protein
CNS	Central Nervous System
CTs	Combination therapies
CYT	Cytochrome P450
DEAQ	N-desethylamodiaquine
DHA + PQ	Dihydroartemisinin–Piperaquine
G6PD	Glucose -6- Phosphate dehydrogenase
GST	Glutathione S-transferases
HIV	Human immunodeficiency virus infection
MDR	Multidrug transporter
OATP	Organic anion transporting peptide
OCTs	Organic cation transporters
PfMDR	*P. falciparun* Multidrug Transporter
P-gp	P-glycoprotein
QNM	Quinoneimine
SLCO1B1	Solute carrier organic anion transporter
SP	Sulfadoxine–Pyrimethamine
UGT	UDP Glucuronosyltransferase
W.H.O	World Health Organization

Introduction

The Malaria parasite is a eukaryotic single-celled pathogen that causes malaria in tropical countries. *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malaria,* and *Plasmodium knowlesi* are the five *Plasmodium* species that cause malaria in humans. The bite of infected female mosquito vectors of more than 30 anopheles mosquito species transmits parasites to humans. The malaria parasite replicates in the red blood cells of its human host after an initial replication phase in the human liver. These blood-stage replicating phases can last for weeks or months in infected people, causing typical illness symptoms including fever and anemia and leading to organ damage and death (Aminake and Pradel 2013).

The incidence of malaria has declined substantially from 80/1000 in 2000 to 58/1000 in 2015; the decline has paused, with 2019 registering 57/1000. The age profile of the disease is also changing, most likely due to reductions in natural immunological protection, particularly in Africa (Pernaute-Lau et al. 2022). According to the World Health Organization (W.H.O), the number of malaria cases in Sub-Saharan Africa may double, since efforts to combat the endemic disease have been hampered by the coronavirus outbreak (WHO 2020; Audu et al. 2021).

Current malaria disease countermeasures include long-lasting insecticidal nets, indoor residual spraying, prophylactic medications, and untreated nets (Wangdi et al. 2018); after malaria has established itself in the host, the primary method of combating the disease is chemotherapy, as there is no effective vaccination (Aminake and Pradel 2013).

Antimalarial Drugs

Antimalarials in widespread use are quinolines (quinine, chloroquine, mefloquine, amodiaquine, and primaquine), antifolates (pyrimethamine, sulfadoxine and proguanil, and artemisinin derivatives (artemisinin, artemether, arteether, and artesunate) (Saifi et al. 2013). This class of agents exhibits a wide variety of pharmacokinetic features. Most of them are well absorbed orally and are primarily eliminated in the urine. A few are also given parenterally, particularly in severe malaria cases (Scholar 2007). To cure an infection, the treatment must be administered into the bloodstream in sufficient amounts to kill the parasites while avoiding substantial unfavorable effects (Aminake and Pradel 2013). Malaria treatment techniques vary depending on the species and severity of the sickness (White et al. 2014).

Typically, antimalarial doses are standardized depending on age and weight; nevertheless, variations in pharmacokinetic characteristics and pharmacodynamic reactions, in pregnant women and children, may need distinct dosing treatments (Wilby and Ensom 2011). Prophylaxis, *falciparum* malaria, and non-*falciparum* malaria management are the three ways antimalarials are used. Visitors from

industrialized countries who travel to malaria-endemic areas exclusively utilize antimalarial prophylaxis (Saifi et al. 2013).

Malaria endemic areas where other infectious diseases, such as HIV infection are common, both treatments can occur concurrently. This may make antimalarials vulnerable to drug interactions, reducing therapeutic efficacy (Kiang et al. 2014). Antimalarial drug resistance is a big concern. Chloroquine and other anti-*P. falciparum* drugs are no longer effective (Packard 2014). Similarly significant treatment failures are frequently observed when sulfadoxine–pyrimethamine (SP) or mefloquine are used (Gatton et al. 2004; Price et al. 2004). The emergence of parasite resistance to most of the existing antimalarials in Africa is limiting the success of malaria treatment. As a result, the WHO has advised that antimalarial treatments be used as combination therapies (CTs) rather than monotherapies. The most favored choice is a combination with unmatched pharmacokinetics (i.e., a short half-life artemisinin and a long half-life companion medication) (Abolaji et al. 2013). Additionally, artemisinin resistance has also been identified throughout South Asia (Ashley et al. 2014). The primary cause of antimalaria resistance is seen to be treatment failure in association with residual medication levels found in malaria-endemic regions (Pongtavornpinyo et al. 2008).

Artemisinin-based combination therapies (ACTs) are the first-line therapy option for uncomplicated *P. falciparum* malaria worldwide (Pernaute-Lau et al. 2022). At the moment, the WHO recommends some of the following artemisinin-based combinations: artesunate–amodiaquine (AS+AQ); artesunate–mefloquine (AS+MQ); artesunate–sulfadoxine–pyrimethamine (AS+SP); artemether–lumefantrine (AL); and dihydroartemisinin–piperaquine (DHA + PQ) (WHO 2021), although mefloquine is now indicated for the treatment of uncomplicated *falciparum* malaria in conjunction with artesunate (Table 1).

Mode of Action and Metabolism of Antimalarial Drugs

Antimalarial medicines target a wide range of targets and have a variety of modes of action. Many of them, such as chloroquine, amodiaquine, mefloquine, and quinine, work by inhibiting heme in the parasitic feeding vacuole. As a result, they impede hemoglobin polymerization, which can be harmful to the *Plasmodium* parasite. Antagonists of folate, some of the medications in this class, such as pyrimethamine and proguanil, are antifolate agents used to treat malarial illness. They interfere with the parasite's folate metabolism (Aminake and Pradel 2013). Antimalarials create free radicals, such as artemether, which kill the malaria parasite or prevent parasitic electron transport. Primaquine produces reactive oxygen species in the parasite, which can obstruct electron transport. Finally, medications such as doxycycline block protein synthesis in the parasite selectively (Scholar 2007). The antimalarial agent's target differs. Most of these medications are primarily effective against the parasite's erythrocytic stage; however, primaquine is effective against the parasite's hepatic and latent tissue stages. Heme is a frequent target of blood schizoniticides, as is folic acid production and mitochondrial electron transport (Fig. 1).

Table 1 WHO list of recommended antimalarials

	Drug Combinations	Treatment Recommendation
Artemisinin-based combination therapy (ACTs)	Artemether + lumefantrine Artesunate + amodiaquine Artesunate + mefloquine dihydroartemisinin + piperaquine Artesunate + sulfadoxine–pyrimethamine (SP) Artesunate + pyronaridine (currently unGRADEd, anticipated to be updated in 2022)	1. Children and adults with uncomplicated *P. falciparum* malaria should be given one of the following treatments. Pregnant women in the first trimester should not be given any of these treatments. 2. Revised dose recommendation for dihydroartemisinin + piperaquine in young children 3. Reducing the transmissibility of treated *P. falciparum* infections: in low-transmission areas, give a single dose of primaquine with ACT to patients with *P. falciparum* malaria (except pregnant women, infants aged <6 months, and women breastfeeding infants aged <6 months) to reduce transmission. G6PD (Glucose -6-Phosphate dehydrogenase) testing is not required. 4. Travelers who have uncomplicated *P. falciparum* malaria when they return to areas that are not endemic should take ACT. 5. Adults and children with uncomplicated *P. vivax, P. ovale, P. malariae,* or *P. knowlesi* malaria (excluding pregnant women in their first trimester) should be treated with ACT in locations where chloroquine-resistant infections exist.
	Quinine + clindamycin	Pregnant women with uncomplicated Plasmodium falciparum malaria should get 7 days of treatment during the first trimester.
Chloroquine		Adults and children with uncomplicated P. *vivax, P. ovale, P. malariae*, or *P. knowlesi* malaria should be treated with chloroquine in locations where the illness is chloroquine-susceptible, with the exception of pregnant women in their first trimester.
Quinine		Quinine should be given to pregnant women who have chloroquine-resistant *P. vivax* malaria in the first trimester.
Artesunate		Adults and children with severe malaria, including babies, pregnant

(continued)

Table 1 (continued)

	Drug Combinations	Treatment Recommendation
		women in all trimesters, and breastfeeding mothers, should be given artesunate intravenously or intramuscularly for at least 24 h or until they can tolerate oral treatment. Complete treatment with 3 days of ACT once the patient has had at least 24 h of parenteral therapy and can tolerate oral therapy.

Data on WHO list of antimalarial drugs are from WHO 2021)

Chloroquine is metabolized by CYP2C8, CYP3A4, and CYP2D6 to an active metabolite (N-desethylchloroquine), as well as other metabolites (Ducharme and Farinotti 1996). Quinine is metabolized to various metabolites in the liver, with 3-hydroxyquinine being the most significant. The major enzyme involved in the synthesis of 3-hydroxyquinine in vitro has been identified as CYP3A4 (Mirghani et al. 2006). Because there is a strong link between the synthesis of (10R)-11-dihydroxydihydroquinine and losartan hydroxylation, it is possible that CYP2C9 is engaged in this metabolic pathway. CYP3A4 catalyzes the entire creation of 3-hydroxyquinine and 2-quininone, as well as the partial formation of (10S)-11-dihydroxydihydroquinine. The 2-quininone, (10R)-, and (10S)-11-dihydroxydihydroquinine all play minor roles in quinine biotransformation in human liver microsomes in vitro (Mirghani et al. 2002). Hepatic CYP2C8 rapidly converts amodiaquine to N-desethylamodiaquine (DEAQ) and a secondary metabolite, 2-hydroxy-amodiaquine, which are both excreted by the kidneys. Hepatic CYP2C8 converts amodiaquine to (DEAQ), and extra-hepatic CYP1A1 and CYP1B1 degrade it to an unidentified metabolite (Giao and De Vries 2001; Yeung et al. 2011; Li et al. 2002; Kerb et al. 2009) (Table 2).

CYP3A4 breaks down mefloquine into two pharmacologically inactive metabolites: carboxy-mefloquine and hydroxy-mefloquine (Kerb et al. 2009). Lumefantrine is metabolized by CYP3A4/5, resulting in desbutylbenflumetol (DBB) metabolite (Noedl et al. 2001; Pernaute-Lau et al. 2022) (Table 2). CYP2A6, CYP3A4/A5, and CYP3A4 (secondary contribution of CYP2B6 and CYP3A5), respectively, metabolize artesunate, artemether, and arteether to dihydroartemisinins and rapidly inactivated to α-dihydroartemisinin-β-glucuronide by the phase II (UDP glucuronosyltransferase) UGT1A9 and UGT2B7 (Ilett et al. 2002; Kerb et al. 2009). Polymorphic CYP2B6 is responsible for the formation of four inactive metabolites after oral artemisinin administration: deoxyartemisinin, deoxydihydroartemisinin, 9,10-dihydrodeoxyartemisinin, and so-called crystal. CYP3A4 may play a secondary role in compensating for reduced CYP2B6 expression (Svensson and Ashton 1999; Kerb et al. 2009) (Table 3).

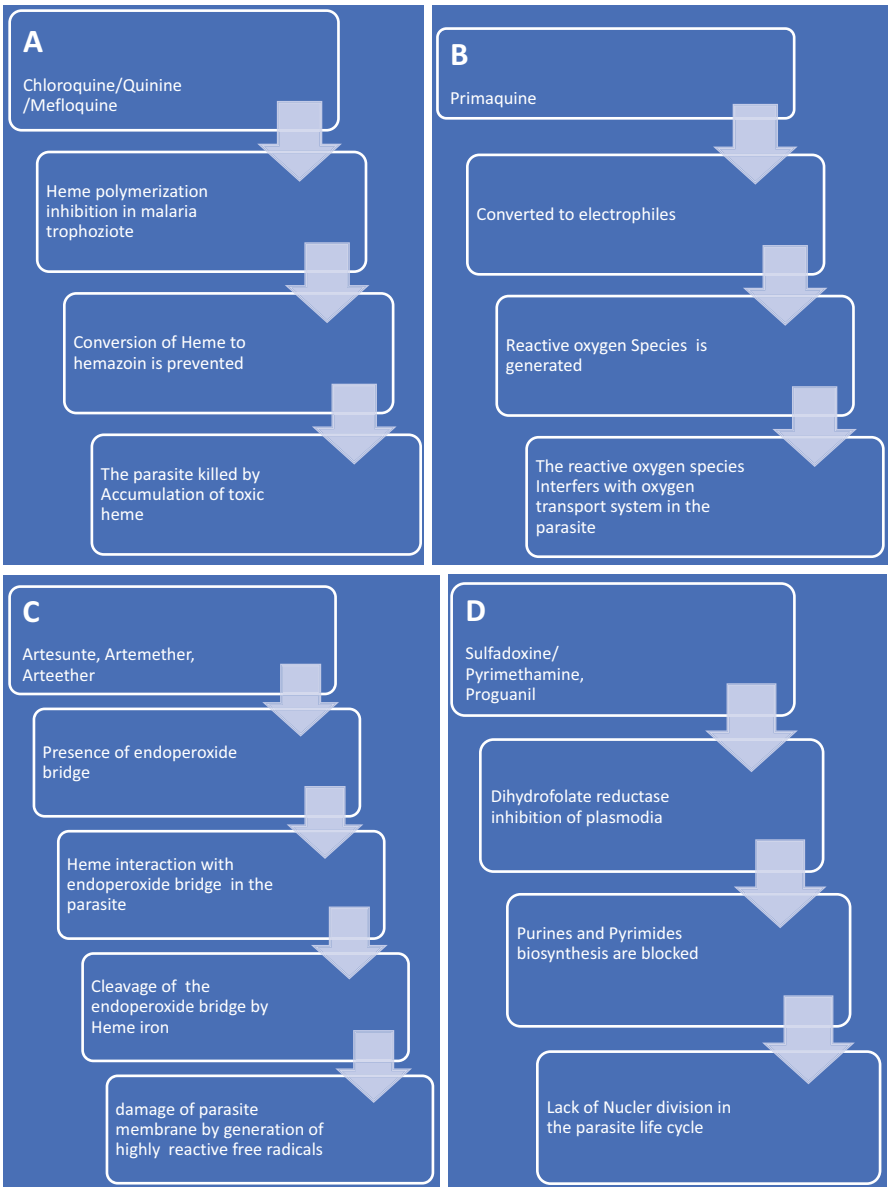

Fig. 1 Mechanism of action behind A: Chloroquine/Quinine/Mefloquine; B: Primaquine, C: Artesunate, Artemether, Arteether; and D: Sulfadoxine/Pyrimethamine. (Data on the mechanism of action of Antimalarial drugs is from (Scholar 2007))

Table 2 Metabolism of quinoline

Artemisinin Drugs	Cytochrome P450 enzymes responsible for metabolism	Metabolite
Quinine	CYP3A4	primary metabolite: 3-hydroxyquinine Minor metabolite: 2-quininone, (10R)- and (10S)-11- dihydroxydihydroquinine
	CYP2C9	(10R)-11-dihydroxydihydroquinine
Mefloquine	CYP3A4	Inactive metabolite: Carboxy-mefloquine Hydroxyl-mefloquine
Lumefantrine	CYP3A4/5	Desbutylbenflumetol
Chloroquine	CYP2C8 CYP3A4 CYP2D6	Active metabolite (N-desethylchloroquine) along with other metabolites ethylchloroquine, 7-chloro-4-aminoquinoline, chloroquine side-chain N-oxide, and chloroquine di-N-oxide
Amodiaquine	CYP3A4	Monodesethy
Piperaquine	CYP3A4 CYP2C8	
Primaquine	CYP3A4 CYP1A2	Carboxy-primaquine

Table 3 Metabolism of artemisinin

Artemisinin drugs	Cytochrome P450, Phase I enzymes	Glucuronosyltransferase, Phase II enzymes	Metabolite
Artesunate, artemether, and arteether	CYP2A6 CYP3A4/5 CYP3A4 CYP2B6	UGT1A9 and UGT2B7	Phase I: active compound: dihydroartemisinin Phase II: inactive metabolites: α-dihydroartemisinin-β-glucoronide
Artemisinin	CYP2B6, CYP3A4 CYP2A6		Inactive metabolites: deoxy-artemisinin, deoxydihydro-artemisinin, crystal-7, 9,10-dihydro-artemisinin

Data on the metabolism of quinoline (Table 2) and Artemisinin (Table 3) are from (Ducharme and Farinotti 1996; Mirghani et al. 2006; Mirghani et al. 2002; Li et al. 2002; Kerb et al. 2009; Noedl et al. 2001; Pernaute-Lau et al. 2022; Ilett et al. 2002; Kerb 2006; Svensson and Ashton 1999)

Toxicity of Antimalarial Drug

"Type A" Toxicity are adverse effects (AEs) caused by overreactions to a medicine; these AEs are foreseeable based on the drug's known effects and are dose or concentration dependent. While "Type B" AEs, on the other hand, are unpredictable based on the drug's known effects; the AE may have an immunological basis because there is frequently no obvious relationship with medicine dose or concentration. Furthermore, some patient categories, such as the elderly, the very young,

(G6PD)-deficient persons, and HIV-positive people, are at a higher risk of severe AEs, and these may be underrepresented in regulatory submissions. Toxicity can range from minor to severe, and it can be reversible or irreversible (Winstanley et al. 2004; Alkadi 2007).

Despite the availability of a variety of antimalarial drugs, malaria remains a deadly disease in tropical places (Abolaji et al. 2013). Antimalarial drugs have a range of effects. The gastrointestinal tract, the eyes, the central nervous system, and the cardiovascular system are all affected by this therapeutic class.

Chloroquine has been shown to cause itching and aggravate psoriasis, reduced myocardial function, hypotension, vasodilation, cardiac arrhythmias, and cardiac arrest among cardiovascular consequences. Confusion, convulsions, and coma are all signs of a central nervous system problem. Chloroquine is hazardous to the cardiovascular system in three ways: it stabilizes the membrane, has direct negative inotropic impacts, and causes direct arterial vasodilation. Antimalarials have a greater cardiotoxicity in patients with acute renal failure, particularly after 3 days of treatment. Chloroquine's reduced toxic metabolite can cause severe eye damage (Alkadi 2007).

When administered in maximum therapeutic doses, quinine causes a trio of dose-related toxicities, including cinchonism, hypoglycemia, and hypotension. Hypotension can arise because of myocardial depression, vasodilation, or dysrhythmia due to quinine's -adrenergic inhibiting action. Patients with G6PD deficiency may develop hemolytic anemia when quinine is administered. Quinine has been observed to inhibit high-tone auditory responses (Alkadi 2007). A significantly high plasma quinine concentration results in auditory complaints, gastrointestinal problems, vasodilation, perspiration, and headache (Bateman and Dyson 1986).

Mefloquine can produce cardiac, gastrointestinal, and CNS AEs, as well nausea, weird dreams, seizures (rarely), and psychosis. Seizures and hallucinations affect one out of every 10,000 people who use mefloquine for chemoprophylaxis. Milder CNS effects (such as dizziness, headache, sleeplessness, and vivid nightmares) are more common, with up to 25% of patients using the drug (Phillips-Howard and ter Kuile 1995). Mefloquine's neuropsychiatric adverse effects include anxiety and paranoia, as well as sadness, hallucinations, psychotic behavior, and perhaps suicide (Alkadi 2007). Mefloquine has gastrointestinal side effects and neuropsychiatric effects ranging from mild anxiety to major neurological and psychiatric adverse events, including psychosis, toxic encephalopathy, convulsions, and "acute brain syndrome" (Lee et al. 2017).

In G6PD-deficient people, primaquine can induce hemolytic anemia or even death, but mild anemia, cyanosis (methemoglobinemia), and leucocytosis are less prevalent. Methemoglobinemia and leukopenia are common side effects of high doses (Hall et al. 1986). Granulocytopenia and agranulocytosis are uncommon AEs of treatment that are typically related to primaquine overdosage. Additional uncommon consequences include hypertension, arrhythmia, and central nervous system symptoms such as despair and confusion (Alkadi 2007). The most significant side effect of primaquine is oxidative hemolysis (Ashley et al. 2014).

Patients receiving amodiaquine for prophylaxis developed hepatitis (hepatotoxicity) and agranulocytosis, and the medicine is no longer advised for this use (Winstanley et al. 2004). Malaria can be treated with pyrimethamine-sulfadoxine. Due to the AEs profile, it is no longer regarded a first-line medication for prophylaxis. Pyrimethamine antimalarial dosages alone induce only minor toxicity, such as skin rashes and a reduction in hematopoiesis. Excessive doses cause megaloblastic anemia. It causes erythema multiforme, Stevens-Johnson syndrome, toxic epidermal necrolysis, dermatitis, serum sickness-like responses, and hepatitis in both adults and children. Hypersensitivity responses may be triggered by toxic sulfamethoxazole metabolites (Alkadi 2007). Antimalarial medication toxicity is one side of the risk-benefit ratio and is evaluated differently based on whether the medical reason for drug uptake is malaria therapy or prophylaxis (Taylor and White 2004).

Biomarkers of the Toxicity of Antimalarial Drugs

Cytochrome P450

The clinical manifestations of genetic diversity in drug-metabolizing enzymes can include the following: effective drug overdose in individuals unable to adequately remove an active substance, inability to convert an inactive prodrug to its pharmacologically active metabolite, and lack of therapeutic efficacy in patients who metabolize an active drug too rapidly or cannot convert an inert prodrug to its active metabolite (Grant 2017).

Cytochrome P450 (CYP) enzymes are polymorphic on a genetic level (Zanger et al. 2008). The human genome has 57 cytochrome P450 (CYP) genes, with isozymes from the families CYP1, CYP2, and CYP3 responsible for the majority of phase I biotransformation of pharmaceuticals and other foreign substances in the human liver (Lewis 2004; Zanger et al. 2008). Polymorphisms in genes encoding drug-metabolizing enzymes are connected to individual differences in drug response (Shah 2005; Marwa et al. 2014).

It is critical to mention that poor metabolizers will metabolize the medication more slowly, resulting in higher plasma concentrations of the medication, which may cause toxicity. Additionally, when prodrugs that require bioactivation are delivered, weak metabolizers may experience therapeutic failure (Zanger et al. 2008). Furthermore, as a result of subtherapeutic drug concentrations, CYP polymorphisms may cause toxicity (in slow metabolizers) and the creation or propagation of drug resistance (in extensive metabolizers) (Marwa et al. 2014) (Table 4).

Despite the lack of research on the influence of the CYP2C8 polymorphism on chloroquine metabolism, clinical implications are unlikely because both the parent drug and its metabolite are pharmacologically active (Gil et al. 2007; Kerb et al. 2009). Although there is a relationship between CYP2C8*2 and *3 and gametocytemia and low parasitemia clearance rates in chloroquine/primaquine-treated people (Sortica et al. 2016).

Table 4 Antimalaria cytochrome P450 polymorphism, their physical expression, and their frequency distribution in people

	Phenotype	Phenotype frequency in African people (%)	Phenotype frequency in Asian people (%)	Phenotype frequency in white people (%)	Antimalarial substrates (minor substrates)
CYP2A6[a]					
*1B (3' UTR gene conversion)	UM[b]	0	7	4	Artesunate (artemisinin)
*2 (p.Leu160His)	PM	0–1	0	1–5	
*4 (hybrid-deleted allele)	PM	1–2	10–24	0–4	
*7 (p.Ile471Thr)	PM	0	6–10	0	
*10 (p.Ile471Thr/p.Arg485Leu)	PM	0	2–4	0	
*17 (p.Val365Met)	PM	9–10	0	0	
Total frequency of PM phenotypes		2	4–12	1	
CYP2B6					
*4 (Lys262Arg)	UM	0	7	4	Artemisinin (arteether, dapsone)
*6 (Gln172His/Lys262Arg)	PM	25–49	12–19	15–25	
*18 (Ile328Thr)	PM	2–8	0	0	
CYP2C8					
*2	PM	11–17	0	<1	Amodiaquine, chloroquine
*3	PM or UM[c]	0.4–2.1	<0.1	15	
Total frequency of PM phenotype:		1.5–4	<0.1	2	

(continued)

Table 4 (continued)

	Phenotype	Phenotype frequency in African people (%)	Phenotype frequency in Asian people (%)	Phenotype frequency in white people (%)	Antimalarial substrates (minor substrates)
CYP2C9					
*2	PM	2–4	0	10–15	Dapsone
*3	PM	1–2	1–4	5–10	
*5	PM	1–2	0	0	
*6	PM	0.6–3	0	0	
*8	PM or UM (substrate dependent)	3–9	0	0	
*11	PM	2–5	0	<1	
Total frequency of PM phenotypes		1–6	<1	2–6	
CYP2C19					
*2	PM	10–25	20–45	13–19	Chlorproguanil, proguanil
*3	PM	0–2	5–13	<1	
*17	UM	18	18	4	
Total frequency of PM phenotypes		2–7	10–30	2–5	

CYP2D6					
*3	PM	<1	0.8–1	1–3	(Chloroquine)
*4	PM	1–8	0.5–1	12–23	
*5	PM	1–7	5–6	2–7	
*6	PM	<1	–	0.7–1.4	
*10	IM	3–9	39–70	2–8	
*17	IM	9–34	–	<1	
*29	IM	5–7 (20 in people from Tanzania)	–	<1	
*1/2xN	UM	2–5 (29 in people from Ethiopia)	0.5–1	1–2	
Total frequency of PM phenotypes		1–3	1–2	2–10	
CYP3A5[d]					
*3	PM[e]	12–40[f]	60–75	85–95	Artelinic acid, artemether, chloroquine, dapsone, halofantrine, lumefantrine, mefloquine, primaquine, quinine, quinidine (artemisinin, arteether,[g] proguanil, chlorproguanil, clindamycin[h])
*6	PM[g]	22	0	0	
*7	PM[g]	10	0	0	
UGT1A1					
*28 [TA]7	PM	39–50	6–13	30–40	
*36 [TA]5	UM	2–7	0	0.2–3	
*37 [TA]8	PM	3–9	0	<1	
*6	PM	0	12–20	<1	
Total frequency of PM phenotypes[i]		10–30	<5	3–10	

(continued)

Table 4 (continued)

	Phenotype	Phenotype frequency in African people (%)	Phenotype frequency in Asian people (%)	Phenotype frequency in white people (%)	Antimalarial substrates (minor substrates)
UGT1A9					
*3 (p.Met33Thr)	PM or UM (substrate dependent)	<1	–	2–4	Dihydroartemisinin
c.-275/-2152 (promoter)	UM	–	0	3–6	
Total frequency of PM phenotypes		<0.1	–	≤1	
UGT2B7					
*2 (p.H268Y)	PM or UM (substrate dependent)	21–32	25–27	46–50	Dihydroartemisinin
Total frequency of PM phenotypes		4–10	6–7	20–25	

NAT2					
*5 (c.341T>C; p. Ile114Thr)	PM	13–51	0.3–11	42–54	Dapsone
*6 (c.590G>A; p.Arg197Gln)	PM	13–37	19–39	24–31	
*7 (c.857G>A; p.Gly286Glu)	PM	<1–7	8–19	1–3	
*14 (c.191G>A; p.Arg64Gln)	PM	2–13	0	<1	
Total frequency of PM phenotypes		32–80	27–69	73–89	

Taken with permission from (Kerb et al. 2009)
UM ultrarapid metabolizer; *PM* poor metabolizer; *IM* intermediate metabolizer
[a]The relevance of CYP2A6 has primarily been shown for nicotine metabolism and tobacco smoke-related disease
[b]Moderately (about 20%) increased activity
[c]CYP2C8*3 metabolic capacity has been shown to be increased for repaglinide and rosiglitazone, for example
[d]Since CYP3A4 and CYP3A5 have largely overlapping substrate specificity, CYP3A4 might compensate lacking CYP3A5 activity for many drugs
[e]Low protein expression with residual CYP3A5 activity
[f]12–15% in African populations, 30–40% in African-American populations
[g]Null alleles causing absence of CYP3A5 protein
[h]Mainly substrates of CYP3A4
[i]Rate of occurrence of Gilbert's syndrome (benign juvenile hyperbilirubinemia)

Compared to CYP2C8 wild-type (*1), amodiaquine incubation utilizing human liver microsomes and recombinant cells, the most common expressed CYP2C8 gene polymorphisms of CYP2C8*2 and CYP2C8*3, CYP2C8*2 resulted in a 50% decrease in metabolic activity and CYP2C8*3 85% decrease in metabolic activity (Kerb et al. 2009). Amodiaquine AEs were more frequent in those with decreased CYP2C8 metabolism (Elewa and Wilby 2017). In 2007, the effect of CYP2C8 on the efficacy and safety of amodiaquine was evaluated in 275 individuals from Burkina Faso. While CYP2C8 genetic variations were not linked with efficacy results in the study, they were associated with an increased risk of AEs (self-reported stomach discomfort) among CYP2C8*2 carriers compared to the wild-type. The same study examined amodiaquine metabolism in recombinant CYP2C8 enzymes and discovered that CYP2C8*2 had a sixfold lower intrinsic clearance than CYP2C8*1 and a significant drop in metabolic activity in CYP2C8*3 (Parikh et al. 2007).

Amodiaquine A CYP2C8*2/*2 carrier, already with decreased enzyme activity, would be converted into a CYP2C8*3 phenocopy via drug–drug interactions, resulting in an increased risk of AQ-associated AEs. In patients with the CYP2C8 poor metabolizer genotype, a slower conversion of amodiaquine to DEAQ may predispose them to generate this highly reactive intermediate, hence increasing the risk of toxicity (Pernaute-Lau et al. 2022; Yusof and Hua 2012).

Artesunate and amodiaquine at a fixed-dose combination or as a loose-dose combination with a 60-day washout period between the two treatments resulted in CYP2A6 ultra-metabolizers (CYP2A6*1/*1B, CYP2A6*1B/*1B, CYP2A6*1B/*8) to have a higher rate of adverse events recorded per individual. Increased liver enzymes, flushing, dizziness, nausea, headache, and epigastric discomfort are all possible AEs (Elewa and Wilby 2017).

CYP2C8*3 (white poor metabolizer allele) significantly decreases amodiaquine metabolism compared to CYP2C8*2 (African poor metabolizer allele). As a result, genotyping for CYP2C8*2 in Africans and CYP2C8*3 in whites may be a potential technique for identifying persons at elevated risk of severe adverse drug responses associated with amodiaquine and allowing for the initiation of alternate medication treatments in these patients (Gil et al. 2007; Kerb et al. 2009). ATS-AQ medication has been seen to increase the prevalence of AEs in CYP2A6*1B carriers (Yusof and Hua 2012).

Lumefantrine has been shown to be a CYP2D6 inhibitor in vitro. This may also facilitate interactions, which may be increased by certain pharmacogenetic profiles (Pernaute-Lau et al. 2022). Although CYP2C19 variations altered proguanil metabolism, no difference in effectiveness was detected. High concentrations of proguanil were detected in poor metabolizers, but cycloguanil was detected more frequently in extensive metabolizers (Elewa and Wilby 2017).

Using recombinant enzymes (fluorometric test) and human liver microsomes (LC–MS/MS analysis), the structural analogs artemether, artesunate, and dihydroartemisinin of artemisinin were evaluated for their effects on seven key human liver CYP isoforms (CYP1A2, 2A6, 2B6, 2C9, 2C19, 2D6, and 3A4), and this revealed reduced enzymatic activity of CYPs, primarily by a mixed mechanism of inhibition, particularly CYP1A2, 2B6, 2C19, and 3A4. A substantial likelihood of

interaction was predicted in vivo if artemisinin was given with CYP1A2 or 2C19 substrates (Ericsson et al. 2014).

UGT1A9*3 and UGT2B7*2 which depend on the substrate for phase 2 metabolism of Dihydroartemisinins are either Poor or Ultrarapid metabolizers (Kerb et al. 2009). Various antimalarial metabolizing enzymes polymorphism, their phenotype expression, and significance of their genetic variability are provided in Table 4. Genetic variation of enzymes involved in ACT metabolism, and its impact on the efficacy and likely link AEs should be a new area of further experimental investigation.

Drug Transporter

Drug transporters can be classified as uptake and efflux transporters. Uptake transporters function by assisting medications in their transfer into cells. The organic anion transporting polypeptide (OATP, SLCO) family and organic cation transporter (OCT, SLC22A) families are all included in this class of transporters. Efflux transporters, on the other hand, act to transfer medicines from the intracellular to the extracellular milieu, frequently against steep concentration gradients, and members of the P-glycoprotein (ABCB) family, such as multidrug resistance protein 1 (MDR1), belong to this class of transporters (Ho and Kim 2005).

P-Glycoprotein

Drug transporters are becoming more well recognized as a critical factor affecting drug distribution and reaction (Kerb 2006). The multidrug transporter (MDR) P-glycoprotein (P-gp), which confers on cells the ability to resist deadly doses of some cytotoxic medications by pumping the chemicals out of the cells and thereby lowering their cytotoxicity, is one significant mechanism of MDR. P-gp is a member of the ATP-binding cassette (ABC) family of transporter molecules, which rely on ATP hydrolysis to function (Lehne 2005). The ATP-dependent efflux transporter MDR1 (ABCB1, P-glycoprotein) is now generally recognized as having a considerable impact on drug absorption and excretion in organs such as the gastrointestinal tract, liver, and kidney. Furthermore, MDR1 expression at the blood–brain barrier prevents many medications from entering the central nervous system. Given MDR1's importance in the drug disposition process, it is not unexpected that single nucleotide polymorphisms (SNPs) in this transporter are being investigated as a potential determinant of individual drug response (Kerb 2006), as drug transporter gene polymorphisms are linked to individual differences in drug responsiveness (Shah 2005).

PfMDR1 and PfMDR2 are two *P. falciparum* genes that are similar to the mammalian multiple drug resistance (MDR) gene (Wilson et al. 1989; Gómez-Saladín et al. 1999). Chloroquine resistance has been linked to mutations in the PfMDR1 gene in some *P. falciparum* isolates (Gómez-Saladín et al. 1999). A study

has shown long-term chloroquine treatment causes MRP1 overexpression and chloroquine resistance (Oerlemans et al. 2006). One study used Caco2 cells to identify chloroquine as a substrate and another as a non-substrate inhibitor for human MDR1 (Hayeshi et al. 2006). Chloroquine is both an ABC transporter inhibitor and a possible substrate for some ABCs. As a result, transporters appear to be interesting targets, especially in the case of chloroquine-induced CNS adverse pharmacological effects, which have been linked to drug interactions and pharmacogenetic susceptibility (Alisky et al. 2006; Kerb et al. 2009). After inhibiting P-glycoprotein with cyclosporine, verapamil, or mefloquine, MDR1a-knockout mice and wild-type mice had higher quinine concentrations in their brains (Pussard et al. 2007; Kerb et al. 2009). Quinine is a substrate for MDR1 P-gp, in in vitro research. Not only did drug interactions result in metabolic interactions, but they also resulted in enhanced quinine uptake by P-gp-protected tissues. While such interactions may have consequences for the advancement of chemotherapy, they should also be considered in light of the possibility of enhancing undesirable effects (Pussard et al. 2007).

In individuals having ABCB1 T variations, decreased MDR1 expression results in decreased mefloquine efflux from the brain, exposing individuals to high tissue concentrations associated with neuropsychiatric symptoms. This finding may suggest a critical function for local MDR1 expression at the blood–brain barrier in causing mefloquine to accumulate in the brain without causing systemic exposure. Elacridar boosted mefloquine brain concentrations without affecting blood levels, whereas loss of P-glycoprotein activity in MDR1a knockout mice increased digoxin (an MDR1 substrate) brain concentrations approximately 40-fold while doubling plasma levels. Nonetheless, the findings of this little study must be interpreted cautiously, as the study was with small numbers of people and a high drop-out rate of 40% (Fromm et al. 1999; Kerb et al. 2009).

Mefloquine interacts with MRP1 and MRP4 in human red blood cells, and is likely to act not only as an inhibitor of these proteins but also as a substrate for them (Kerb et al. 2009). MDR1 1236TT, 2677TT, and 3435TT genotypes, as well as the 1236-2677-3435 TTT haplotype, have been linked to the neuropsychiatric side effects of mefloquine in women. MDR1 polymorphisms may have a key role in predicting the occurrence of mefloquine's neuropsychiatric side effects, especially in female travelers (Table 5) (Aarnoudse et al. 2006). Drug transporter gene polymorphisms linked to recent individual antimalarial drug toxicity need further investigation.

Organic Anion Transporter 1B1

The organic anion transporting peptide OATP1B1 is encoded by the SLCO1B1 gene, which has genetic variation and is an example of a drug transporter that raises plasma levels of many medicines by reducing liver uptake (Kerb 2006). Quinine could be a substrate for OCTs in humans. Since OCT1 is nearly exclusively expressed in the liver. Hepatic quinine metabolism may be aided by OCT1-mediated absorption, but pancreatic OCT2 may contribute to the quinine-related rise in insulin production (Kerb et al. 2009). Chloroquine was described as an important inhibitor

Table 5 Drug transporter involved with antimalaria use

	Polymorphism	Phenotype	Phenotype frequency in African people (%)	Phenotype frequency in Asian people (%)	Phenotype frequency in white people (%)
Efflux transporters					
ABCB1 (MDR1)					
Liver, intestine, kidney, brain	c.1236C>T	Reduced function or expression	21	69	46
	c.2677G>T/A (p. Ala893Ser)	Higher digoxin concentration	<1–3/0[a]	45/7	46/2
	c.3435C>T	Higher digoxin concentration	10–16[a]	40	56
ABCG2 (BCRP1)					
Liver, intestine, brain	c.34G>A (p. Val12Met)	Controversial data	4–6	15–17	2–10
	c.421C>A (p. Gln141Lys)	Reduced expression or function	1–5	26–35	9–12
Uptake transporters					
SLCO1B1 (OATP1B1)					
Liver	c.521T>C (Val174Ala), *5/*15	Reduced function; higher pravastatin, atorvastatin, or rosuvastatin concentrations	1–2	11	12–15

(continued)

Table 5 (continued)

	Polymorphism	Phenotype	Phenotype frequency in African people (%)	Phenotype frequency in Asian people (%)	Phenotype frequency in white people (%)
SLC22A1 (OCT1)					
Liver	c.1201G>A (p.Gly401Ser)	Reduced function, higher metformin concentration, reduced effect	1	0	1–3
	c.1258–1260delATG (p.Met420del)	Reduced function, higher metformin concentration, reduced effect	3	0	16–19
	c.1393G>A (p.Gly465Arg)	Reduced function, higher metformin concentration, reduced effect	2	0	<1
SLC22A2 (OCT2)					
Kidney, other organs (not liver)	c.808G>T (p.Ala270Ser)	Reduced function, reduced renal metformin clearance	11	9	16

Taken with permission from (Kerb et al. 2009)

[a]Increasing prevalence from south to north

of OATP1A2 and OATP1B1 functions, representing a possible role in drug–drug interactions and malaria treatment (Sortica et al. 2017). The various family of drug transporter, its tissue distribution, the important role it plays, polymorphism, and various physical expression are shown in Table 5.

Glucose-6-Phosphate Dehydrogenase

G6PD deficiency is passed down through the generations as an X-linked characteristic (Adam 1961). G6PD deficiency can cause hemolytic anemia when exposed to external stimuli or medicines that cause oxidative stress, which the red blood cells cannot withstand, causing them to break down. The severity of hemolytic anemia is determined by the drug's dose, the degree of G6PD deficiency, as well as the age of erythrocytes (Luzzatto and Seneca 2014).

G6PD enzymatic activity is seen to be reduced in individuals who suffer from hemolysis caused by primaquine. Several studies have shown a link between hemolytic anemia and the usage of primaquine in G6PD-deficient patients over the years. G6PD (A-) individuals treated with chlorproguanil–dapsone–artesunate had a risk ratio of 10.2 compared to G6PD normal individuals, with the onset of hemolytic anemia and a decrease in hemoglobin levels, according to a trial comparing amodiaquine–sulfadoxine–pyrimethamine to chlorproguanil–dapsone–artesunate (Elewa and Wilby 2017). Another study comparing the effects of chlorproguanil–dapsone–artesunate against artemether–lumefantrine on children's hemoglobin levels showed that 35% of children with low hemoglobin levels were G6PD deficient (Premji et al. 2009).

In a study, two of 20 children deficient in the G6PD enzyme who were treated with chloroquine were reported to have experienced hemolysis when concurrently taking chloramphenicol, which is known to cause hemolysis in G6PD deficiency (Choudhry et al. 1990), although there is no indication that chloroquine monotherapy is associated with hemolytic anemia (Youngster et al. 2010). Patients with G6PD deficiency may develop hemolytic anemia, when quinine is administered (Alkadi 2007). 702 children with acute uncomplicated *P. falciparum* malaria were treated with chlorproguanil-dapsone+artesunate (CD + A) and (AQ + SP) in a secondary analysis of an open-label randomized study. The hematocrit of patients with G6PD deficiency who received CDA was considerably lower. Patients with G6PD deficiency had a greater risk of severe anemia after receiving CDA or AQ+ SP therapy than those who did not have the deficiency (Fanello et al. 2008).

Glutathione S-Transferase

Glutathione S-transferases (GSTs) are a set of enzymes that catalyze the conjugation of glutathione (GSH) to numerous endogenous and exogenous substances. The removal of xenobiotics is aided by the action of these enzymes (Valko et al. 2007). Amodiaquine and DEAQ secondary effects have been linked to metabolic bioactivation toward quinonimine (QNM) reactive metabolites in animal studies

and in vitro techniques (Pernaute-Lau et al. 2022; Harrison et al. 1992). QNMs are detoxified via the glutathione system, most likely via Phase II polymorphic glutathione S-transferases (GST), resulting in the formation of QNM-GS conjugates (Pernaute-Lau et al. 2022; Shimizu et al. 2009). GSTP1, GSTA4, GSTM4, GSTM2, and GSTA2 have been proposed as possible participants (Pernaute-Lau et al. 2022; Zhang et al. 2017). Consistent with these studies, chronic use of Amodiaquine at high doses or for an extended period may result in glutathione depletion and ultimately liver toxicity.

Potential Applications to Prognosis, Other Diseases, or Conditions

This book chapter has provided an overview of recent advances relating to biomarkers of the toxic effects of chemotherapeutic drugs with a focus on antimalarial. Various gene polymorphisms involved in the metabolism of antimalarial drugs, drug transport proteins, GSTs, and G6PD deficiency are seen to be useful markers in detecting the toxicities of antimalarial drugs. Further research to link specific toxicity of first-line WHO-recommended antimalarial drugs to these markers is highly needed. The value of antimalarial biomarkers continues to expand in all areas of clinical practice, and they are beneficial at every stage of patient care, whether they are used to forecast, diagnose, or monitor toxicity to both the monotherapy and ACTs. While illness symptoms are subjective, biomarkers provide an objective, quantifiable method for characterizing antimalarial treatment toxicity.

Summary

- Antimalarial drugs may cause several toxicities.
- Genetic variations of CYP were associated with decreased enzyme activity and increased risk of AEs.
- Poor metabolizer genotype and a slower metabolism may predispose an individual to generate highly reactive intermediate, hence increasing the risk of toxicity.
- Some antimalarial drugs have been shown to be a CYP inhibitor in vitro. This may also facilitate interactions, which may be increased by certain pharmacogenetic profiles.
- MDR1 polymorphisms may have a key role in predicting the occurrence of toxic effects.

Key Facts of Antimalarial Toxicity

- Malaria parasite replicates in the human host's red blood cells (RBCs). These blood-stage replication phases can last weeks or months in infected individuals, resulting in usual sickness symptoms like fever and anemia and eventually organ damage and death.

- Once malaria has established itself in the host, chemotherapy is the major means of treatment.
- Antimalarials are used in three different ways: prevention, falciparum malaria therapy, and non-falciparum malaria treatment. Antimalarial prophylaxis is virtually exclusively utilized by travelers from developed countries visiting malaria-endemic regions.
- Antimalarial medications can have a variety of adverse effects (AEs). This medicinal class causes toxic effects on the gastrointestinal tract, the eyes, the central nervous system, and the cardiovascular system.
- Individual variability in medication responsiveness may be associated with polymorphisms in genes encoding antimalarial drug-metabolizing enzymes.
- Poor metabolizers will metabolize the antimalarial drug more slowly, resulting in increased medication plasma concentrations, which may induce toxicity.
- Drug transporters are becoming more well recognized as a critical factor affecting drug distribution of antimalarial drug and may cause various AEs
- Antimalarial drugs can cause hemolytic anemia in individuals with Glucose-6-Phosphate dehydrogenase (G6PD) deficiency.

Mini-Dictionary of Terms

Malaria: A disease caused by the *Plasmodium* parasite that is transmitted by female Anopheles mosquitoes.

Antimalaria: Medication used to cure or prevent malaria.

Artemisinin-based combination therapies (ACTs): The first-line therapy in almost all areas where malaria is endemic. An artemisinin derivative and a partner medication are the two components of an ACT.

Cytochrome P450 (CYP): This is a hemoprotein that regulates drug and xenobiotic metabolism.

Drug transporters: Membrane proteins that play a role in drug uptake and efflux in a variety of tissues, including the intestine, liver, kidney, and brain.

G6PD deficiency: This is a genetic condition in which a person lacks enough of an enzyme called G6PD, which aids red blood cell function. When the body is subjected to infection, severe stress, specific medicines, chemicals, or foods, the red blood cells in G6PD deficiency break down.

Toxic Effects: An adverse effect of medicine caused by an amplification of the actors responsible for the therapeutic response

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Part VI

Biomarkers in Models and Modeling Toxicity

Linking Arsenic, DNA Methylation Biomarkers, and Transgenerational Neurotoxicity: Modeling in Zebrafish

47

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Abstract

Epigenetic mechanisms play a crucial role in maintaining nervous system functions, and dysregulation has been related to the development of neurological disorders. The factors that induce these alterations are diverse, but in recent years the role of environmental pollutants has been the subject of intense study. Arsenic (As) is a ubiquitous neurotoxicant in the environment that has been recognized for decades as a deregulator of epigenetic processes inducing DNA methylation modifications in animal models and humans. As can alter methylation at the promoter of neural genes and cause changes in their expression level and potentially could be transmitted to subsequent generations. However, transgenerational toxicity studies with arsenic and other contaminants remain very scarce in the literature. The zebrafish is an emerging model with qualities such as rapid and inexpensive generation and represents an opportunity to increase knowledge about the mechanisms of transgenerational transmission associated with exposure to pollutants. In this chapter, we review some relevant aspects of modeling the transgenerational effect of arsenic by using epigenetic biomarkers associated with DNA methylation. Recommendations are made for obtaining generations; also some specific gene biomarkers of interest are proposed as some cost-effective methodologies for the analysis.

Keywords

Zebrafish · Epigenetics · DNA methylation · Transgenerational · Neurotoxicity · Arsenic · Nervous system

Abbreviations

As	Arsenic
As/L	Arsenic per liter
AS3MT	Arsenite methyltransferase
As^{III}	Trivalent arsenic/arsenite
As^{V}	Pentavalent arsenic/arsenate
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
CpG	Cytosine guanine dinucleotide
Darpp-32	Dopamine- and cAMP-regulated neuronal phosphoprotein
DML	Differentially methylated loci
DMRs	Differentially methylated regions
DNMTs	DNA methyltransferases
ELISA	Enzyme-linked immunosorbent assay
F0	Parental generation

F1/F2/F3	Offspring generations
H3K27ac	Histone 3 lysine 27 acetylation
H3K4me3	Histone 3 lysine 4 trimethylation
HPLC	High-performance liquid chromatography
HPTMs	Histone posttranslational modifications
LC-MS/MS	Liquid chromatography-mass spectrometry
LTP	Long-term potentiation
LUMA	Luminometric methylation assay
MAs^{III}	Monomethylarsenite
MBP	Myelin basic protein
MMA	Monomethylarsonic acid
MSP	Methylation-specific PCR
$NaAsO_2$	Sodium arsenite
PP1	Phosphatase 1
PPP1R1B	Protein phosphatase 1 regulatory subunit 1B
qRT-PCR	Quantitative real-time polymerase chain reaction
RELN	Reelin
SAM	S-Adenosylmethionine
TEI	Transgenerational epigenetic inheritance
Tm	Melting temperature
TrkB	Tropomyosin-related receptor kinase B
5hmC	5-Hydroxymethylcytosine
5mC	5-Methylcytosine

Introduction

Arsenic is a ubiquitous element naturally present in soils. Its presence in various aquifers worldwide suggests that the main route of human exposure is through the use and consumption of arsenic-contaminated water, mainly through the oral route. High As concentrations have been documented in various regions of the world, such as Argentina with 0.2 mg As/L (Concha 1998), Taiwan 0.05–1.98 mg As/L (Yen et al. 2007), and Bangladesh 0.8 mg As/L (Kinniburgh and Kosmus 2002).

Numerous investigations over the decades have demonstrated the negative impact of As exposure on the nervous system of humans. For example, among the described affectations, As can produce encephalopathy, decrease IQ (Calderón et al. 2001; Tsai et al. 2003), and alter cognitive function in humans (Brinkel et al. 2009). In animal models, environmentally relevant doses of As exposure have consistently shown adverse effects on several parameters associated with the nervous system, such as motor function, learning, and memory (Bardullas et al. 2009; Dipp et al. 2018). This chapter provides an extensive review of the effect of arsenic on the nervous system (Rodríguez 2003). On the contrary, studies have been contradictory in humans. Some reports indicate that sustained exposure can be detrimental to cognitive activity in school-aged infants (Rosado et al. 2007), while other work has failed to demonstrate effects in humans (Kordas et al. 2015). They were suggesting the

complexity of As concerning its toxic effect on the nervous system. One possibility for these discrepancies between studies may be due to numerous variables, such as age differences in the assessment of infants, sensitivity of neurobehavioral parameters, and metabolic profile of individuals given that the metabolic profile of methylated As species is influenced by the ethnicity of individuals (López-Carrillo et al. 2014; Yáñez et al. 2015).

In recent years it has been recognized that environmental stressors can produce epigenetic alterations leading to chronic degenerative diseases. The best-studied epigenetic mechanisms are DNA methylation and posttranslational modifications to histones (HPTMs). MicroRNAs have been recognized as another component of the epigenome, which plays a critical role in regulating gene expression to maintain cellular homeostasis (Portela and Esteller 2010).

Epigenetic mechanisms play a crucial role in maintaining nervous system function, as they are involved in neuronal programming during the early stages of embryonic development. In the case of DNA methylation, it has been suggested as a regulator in the formation and maintenance of memory (Du et al. 2018), since methylation changes can occur in specific loci, affecting the expression of neural genes. Currently, evidence shows dysregulation in epigenome and association with the development of neurological diseases (Landgrave-Gómez et al. 2015).

One of the most relevant aspects of epigenome alterations due to exposure to environmental pollutants is the possibility that these changes are preserved in the germline and transmitted through generations. This concept is called transgenerational toxicity and is an emerging topic of study in toxicology. Growing evidence shows that various substances can induce adverse effects across generations (Xin et al. 2015). For example, early studies with bisphenol A and vinclozolin in rodents showed that gestational exposure produces transgenerational changes in the reproductive, endocrine, and nervous systems (Gillette et al. 2014). However, many controversies surround this unconventional form of transmission, partly because the evidence on transgenerational toxicity is a recent issue and studies in the literature are scarce and mainly focused on endocrine disruptors. The focus of this chapter is to review DNA methylation as a biomarker to assess the transgenerational effect of arsenic on the nervous system, taking advantage of the zebrafish model which has proven to be a valuable model to explore changes in DNA methylation status.

Transgenerational Epigenetic Inheritance

Transgenerational epigenetic inheritance (TEI) is the ability to transmit phenotype or gene expression patterns from one generation to another without changes in DNA sequence. TEI-associated mechanisms occur when some regions of the germline epigenome and during embryonic development escape the epigenetic reprogramming process. Additionally, the TEI mechanism can include mutations in genes associated with epigenome regulation systems, such as DNMTs, DNA demethylases, and alterations in the histone methylation mechanism (Blake and

Watson 2016). Thus, although there are likely to be numerous molecular mechanisms involved in TEI, the prevailing hypothesis suggests germline DNA methylation is the most likely candidate.

Role of Arsenic-Induced DNA Methylation in Neurotoxicity

DNA methylation is the most widely studied epigenetic mechanism and was the first recognized mechanism induced by arsenic exposure. Studies associating As exposure with altered DNA methylation are abundant in the literature. For a more in-depth review of this topic, refer to Bailey and Fry (2014). Several studies have unequivocally demonstrated that exposure induces genomic DNA modifications in cellular, animal, and human models (Zhang et al. 2014). In utero As exposure has been associated with the alteration in DNA methylation that increases the risk of developing chronic degenerative diseases in adult stages (Farzan et al. 2013). DNA methylation consists of the transfer of a methyl group to the fifth carbon position of cytosine residues. Aggregation of 5-methylcytosines mainly occurs in the CpG islands, that is, DNA methylation regions in promoters to regulate gene expression through transcriptional silencing.

DNA methyltransferases regulate DNA methylation. In mammals, DNMT-3A and DNMT-3B regulate de novo methylation, while DNMT-1 maintains methylation status. Arsenic-induced dysregulation of genomic DNA methylation can occur by three mechanisms: (1) alteration in the activity of DNA methyltransferase; (2) decrease in DNMT1 and DNMT3A expression; and (3) competition for S-adenosylmethionine (SAM) for the donation of methyl groups (Bailey and Fry 2014).

There are tissue-specific DNA methylation patterns in vertebrates. In particular, nervous tissue conserves a methylation pattern that distinguishes it from nonnervous tissue. For example, neural tissues such as the cerebellar cortex, basal ganglia, and temporal cortex show a higher methylation percentage than other nonneural tissues such as the stomach, liver, and lungs (Ghosh et al. 2010). Moreover, different brain regions show a distinctive methylation pattern which seems to play a crucial factor in the specialization of the brain (Ladd-Acosta et al. 2007). These specific DNA methylation patterns are an aspect to be considered in the context of As exposure. As and its metabolites are distributed heterogeneously among tissues, with the nervous system being one of the primary tissues of accumulation. Also, high levels of methylated arsenic species (MMA) are observed in the hippocampus compared to other brain regions (Li et al. 2020).

Together, these studies suggest that arsenic exposure may produce changes in the DNA methylation pattern, altering gene expression in specialized regions of the brain. In this regard, Martínez et al. (2011) demonstrated that gestational exposure to low doses of As (3 and 36 ppm) induces changes in the methylation pattern on reelin (RELN) and protein phosphatase 1 (PP1) genes in the hippocampus and cerebral cortex. These modifications were accompanied by alterations in learning and

memory functions in adult rodents. The above could support the idea that the mechanisms of epigenome damage by environmental pollutant toxicity may be tissue-specific.

Modeling Transgenerational Arsenic Neurotoxicity in Zebrafish

The zebrafish is an emergent model that has generated considerable interest in toxicology studies. Its advantages are external embryonic development, reduced life cycles, low breeding cost, and small and large clutches that allow high-throughput tests with multiple replicates. However, the limitation in the zebrafish model during experimental design should be addressed. Some model features are presented below in the context of an arsenic poisoning paradigm, although some considerations can be extended to other environmental contaminants.

Arsenic metabolism similarities. Zebrafish share similarities in arsenic metabolism with mammals. More than a decade ago, a family of five aquaglyceroporin genes was identified homologous to the human with the ability to transport water and trivalent arsenicals (As^{III} and MAs^{III}). These transporters are distributed heterogeneously among tissues contributing to arsenic accumulation, mainly in the liver, muscle, and gills (Hamdi et al. 2009). Subsequently, arsenic is biomethylated through the SAM-dependent arsenic methyltransferase enzyme (AS3MT), expressed in all zebrafish tissues. These metabolic pathways generate As species profiles similar to those detected in mammalian tissues, including man (Hamdi et al. 2012). Taken together, the evidence suggests that, from a metabolic perspective, zebrafish have sufficient similarities to mammals to make them viable organisms for modeling arsenic toxicity.

Nervous system similarities. Nervous system organization in zebrafish is comparable to other vertebrates, divided into the forebrain, midbrain, and hindbrain with the spinal cord, cranial, and sensory nerves. Zebrafish neuroanatomy has been examined in detail through its developmental stages and has demonstrated cellular and functional nervous system mechanisms comparable to mammals. In addition, the neurotransmitter systems between mammals and zebrafish show similarities: glutamatergic excitatory, GABAergic inhibitory circuits, and well-defined nuclei of monoaminergic neurons. However, some specific regions in the mammalian brain, such as the hippocampus and substantia nigra, are not present in zebrafish. However, these functions are maintained in the brain by the lateral pallium serving as its functional equivalent. It is recommended to consult a complete atlas on zebrafish neural development in *Atlas of Early Zebrafish Brain Development* (Mueller and Wullimann 2016).

Arsenic exposure methods. The high solubility of inorganic arsenic in water seems to facilitate the exposure pathway in the zebrafish model via direct dosing in the tanks, although there are essential considerations to consider. Trivalent arsenic, usually administered as sodium arsenite ($NaAsO_2$), is highly susceptible to oxidation to arsenate (V), which is considerably less toxic. This has created a problem if working with larvae or adult fish in a chronic intoxication paradigm that

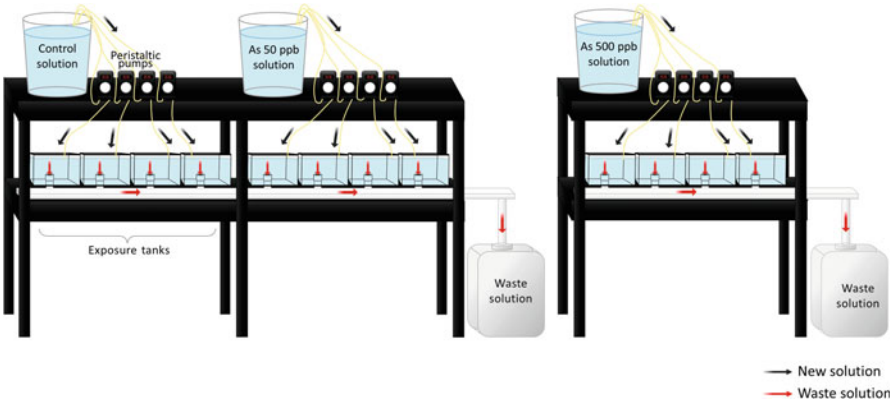

Fig. 1 Continuous flow system for rearing and arsenic exposure of zebrafish. Cost-effective system designed with peristaltic pumps to dispense arsenic solutions from reservoirs; waste solutions are discarded in storage containers for final disposal

requires stable exposure conditions for an extended period. In this case, the most cost-effective recommendation is to build a continuous flow system that does not allow for the recirculation of water from the tanks. Figure 1 shows a cost-effective and easy-to-implement design used in a chronic As poisoning paradigm. In a continuous flow system, the water is replaced in the exposure tanks in an automated way with the help of a peristaltic pump connected to reservoirs with new solutions of As adjusted to pH 7.0. Although practical and economical, this system produces large quantities of waste that must be deposited carefully in a safe place (Dipp et al. 2018).

In contrast, embryonic exposure paradigms simplify these procedures and help model the effect of arsenic during embryonic development. Some studies showed that despite the arsenic being small enough to pass through chorionic pores, the diffusion of arsenic could be affected by this structure (Olivares et al. 2016). On the contrary, other work has shown a toxicity response to arsenic in the whole organism, independent of the presence of the chorion (Coral et al. 2021).

Other issues inherent to the zebrafish model, if working with the developmental intoxication paradigm, are related to the difficulty of labeling individuals. Although methods for individual labeling have been developed (Delcourt et al. 2018), the process has not been as straightforward as in murine models. This limitation makes harder for both exposure monitoring during the development of a single zebrafish individual, and for correlation of the results in its different phases. In addition, visual sexing of zebrafish is only viable until 3 months postfertilization. It is necessary to confirm the sex by examining the gonads due to many studies suggesting that the effect of arsenic has a dependence on sex. This limitation can be confounding if the biomarker studied is sex-biased.

Transgenerational toxicity modeling in zebrafish. The zebrafish is a model that can accelerate the understanding of transgenerational toxicity. It is an affordable and adaptable organism for small laboratories that do not have the infrastructure for

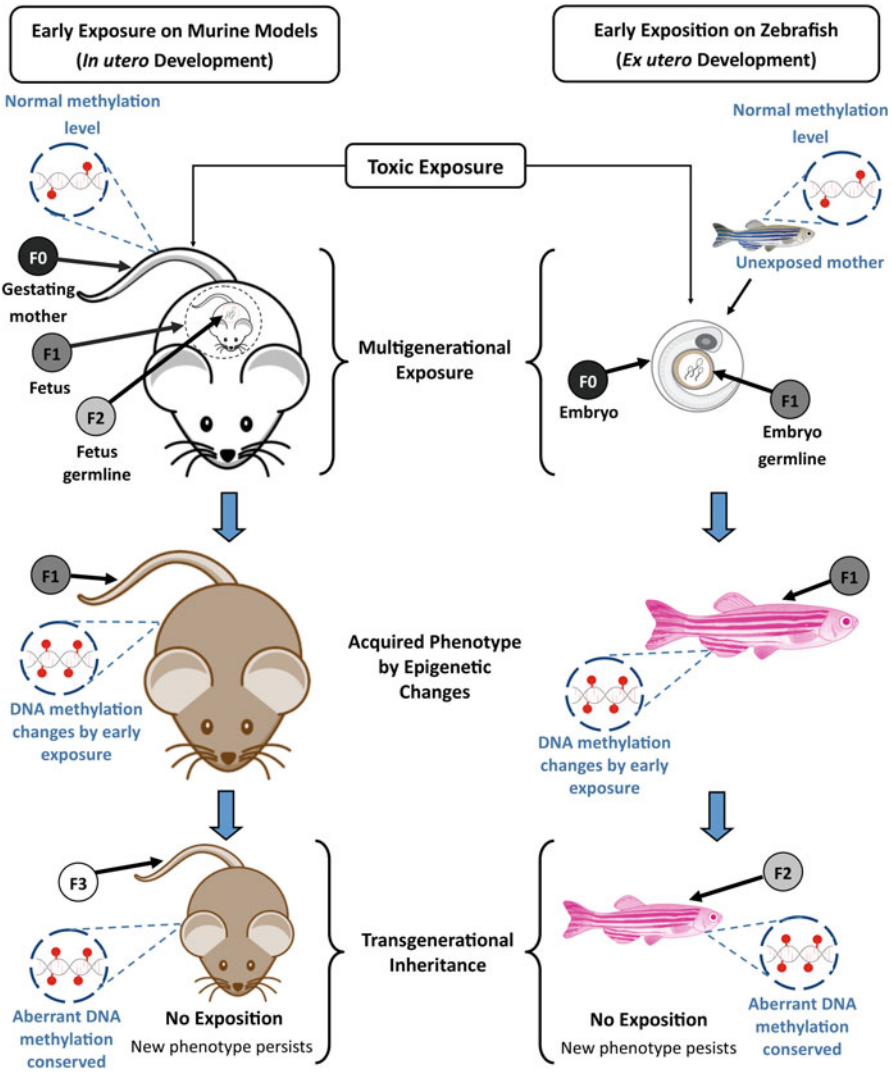

Fig. 2 Transgenerational epigenetic inheritance (TEI) induced by gestational exposure to toxic substances in different models. Exposure to toxic substances during the early stages of embryonic development can lead to phenotypic alterations via DNA methylation changes. Aberrant DNA methylation might be transmitted to subsequent generations, bringing the appearance of the altered phenotype in descendants without continuous exposure to the toxic substance as a transgenerational effect. Early exposure in intrauterine development organisms such as rodents (left) has to be performed by exposing the gestating mother (F0), thus exposing also the fetus (F1) and fetus germline (F2) in a multigenerational exposure. Hence, F2 descendants (F3) will be the first ones without direct contact with the toxic substance, having a transgenerational exposure instead. In organisms with extrauterine development like zebrafish (right), its embryo can be directly exposed to the toxic substance during gestation without involving the mother. Therefore, zebrafish require fewer generations to reach transgenerational exposure than rodents, making zebrafish an ideal model for transgenerational studies

rodents and has the biological characteristics to produce generations in a shorter time. Figure 2 shows a comparison in obtaining generations between rodents and zebrafish. The unexposed or transgenerational generation in zebrafish (F2) is achieved faster than in rodents (F3). Also, the transparent and external development of the eggs is another crucial advantage for tracking toxic effects across generations from the early stages, which is impossible in other models.

However, the phylogenetic distance between fish and mammals leads to differences in DNA methylation mechanisms and needs to be considered in the design of experiments and the interpretation of results. In mammals, total DNA methylation undergoes at least two reprogramming events during the establishment of primordial germ cells. Clearance of the methylome does not occur equally at all sites in the genome. In rodents, methylation is maintained in specific regions of DNA oocytes during the postfertilization stage characterized by global hypomethylation. These regions are known as differentially methylated regions (DMRs).

Consequently, the acquired methylation pattern in the mammalian germline is rarely inherited (Carone et al. 2010). In contrast, in zebrafish the pattern of DNA methylation in the paternal germline is maintained during fertilization and early embryogenesis, suggesting a probable intergenerational transmission of epigenetic memory (Ortega-Recalde et al. 2019). In the adult stage, fish have a high methylation level of 7–8% of all cytosines compared to mammals, although the overall methylation pattern and epigenetic regulatory machinery (Williams et al. 2014).

Affordable Methodologies for the Study of DNA Methylation in Zebrafish

Global DNA Methylation

Global DNA methylation in zebrafish and the rest of vertebrates occurs after adding a methyl group to cytosines, mainly in CG dinucleotides and less frequently in CHG or CHH sequences (Fan et al. 2020). For many years it has been shown that its function is associated with chromatin organization and, consequently, with the repression of non-transcribed genes (Miranda and Jones 2007).

There are a variety of methods to define the global DNA methylation profile in different models. However, in zebrafish, only a few have been used depending on the needs of the research. Considerations vary from the quantity and purity of the DNA to sensitivity and specificity, the availability of necessary equipment and reagents, and cost (Kurdyukov and Bullock 2016). The most used techniques to identify the methylation profile of the whole zebrafish genome include HPLC, mass spectrometry (LC-MS/MS), immunoprecipitation, and LUMA. However, different commercial rapid assessment kits are also based on ELISA assays, with MethylFlash from EpiGentek standing out for use in zebrafish (Teng et al. 2020). ELISA-based assays allow global DNA methylation quantification by binding DNA (100 ng) to high-affinity wells. Then the methylated fractions are detected with antibodies and quantified via colorimetry using a spectrophotometer. These are not only quick

and easy to perform but also have more affordable costs. However, quantification of global methylation using ELISA-based assays is a low-resolution technique, covering a small percentage of the CpG regions and therefore unable to identify the genes methylated by arsenic.

Gene-Specific Methylation

In order to analyze the methylation status of specific loci, several methodologies have been developed in the last few decades. These methodologies detect methylation changes in previously known sequences, such as particular gene promoters, so that they can associate these changes with alterations in the expression of the gene of interest. Most of them are based on bisulfite conversion of genomic DNA as a first step, followed by different downstream methods (Kurdyukov and Bullock 2016).

Sodium bisulfite mediates cytosine deamination, leading to a conversion of unmethylated cytosines to uracil (which will be read as thymines in a subsequent PCR amplification) while methylated cytosines are not modified. Thus, bisulfite conversion allows for the distinguishing of methylation status in a specific residue in a DNA sequence. In techniques such as bisulfite sequencing, bisulfite-treated DNA gets in a PCR assay to amplify a genomic region of interest; amplicon is later isolated and sequenced using NGS (next-generation sequencing), allowing it to recognize methylation-dependent changes on the target region and, hence, potentially determine its original methylation status. However, specialized equipment is required to perform NGS, making bisulfite sequencing assay expensive and challenging to achieve for most laboratories.

Other techniques allow for the measurement of gene-specific methylation levels at considerably lower prices, often using PCR-based methods. One of the main advantages of these techniques is the small amount of DNA needed to perform them. Generally, these methods estimate methylation levels using different primers, each binding a DNA template depending on its methylation status. In the classical protocol methylation specific PCR (MSP), one set of primers for methylated and unmethylated forms of one gene is used. Methylated primers are designed to complement a DNA strand where all cytosines in CpG nucleotides remain as cytosines after bisulfite conversion. In contrast, unmethylated primers complement the same DNA sequence if all cytosines in CpG sites turn into thymines. After amplification, PCR products are analyzed qualitatively or semiquantitatively with electrophoresis gel (Herman et al. 1996).

MSP technology was improved by using quantitative real-time PCR (qRT-PCR) instead of conventional PCR (endpoint). MethyLight technology uses fluorescent probes (such as TaqMan) specially designed to bind to DNA based on its methylation status to perform highly sensitive quantitative analysis of MSP products without gel electrophoresis (Eads 2000). However, the cost of MethyLight protocols

can be high for some small laboratories because specialized fluorescence probes are still considered expensive.

MethySYBR uses SYBR green as a fluorescent dye during qRT-PCR instead of fluorescent probes, which helps perform low-cost gene-specific methylation analysis (Lo et al. 2009) (Fig. 3). Although this method has less sensitivity than MethyLight, some studies have demonstrated the feasibility of this methodology in different models, including zebrafish (Valles et al. 2020). In addition, MethySYBR can incorporate melting curve analysis to determine the methylation level of PCR products by examining their melting temperature (Tm) (Lo et al. 2009).

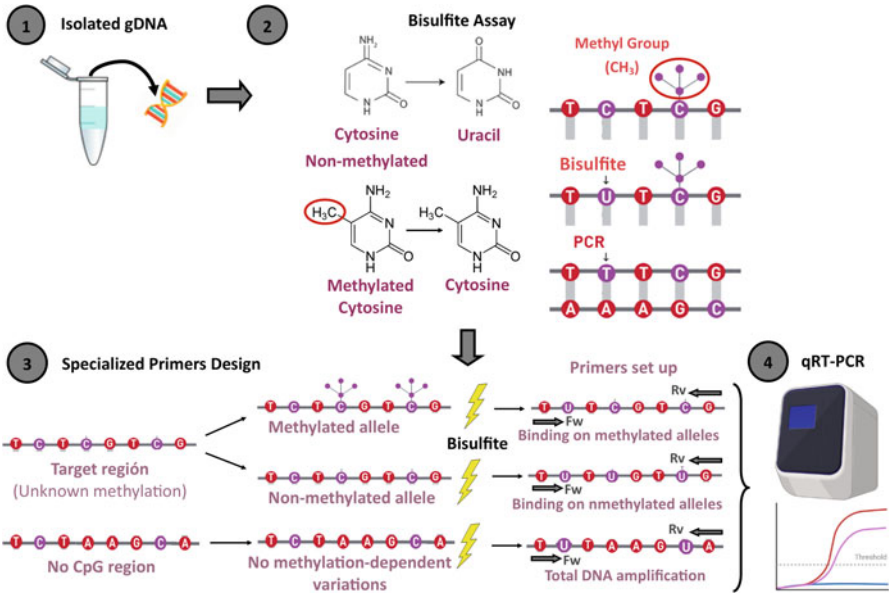

Fig. 3 General basis of MethySYBR assay for gene-specific methylation analysis. Genomic DNA (gDNA) has to be isolated from the tissue of interest (1) for its conversion through bisulfite assay, on which unmethylated cytosines turn into uracil while methylated cytosines remain intact (2). After this, bisulfite-treated DNA is used for a qRT-PCR reaction, on which the different forms of the same target region will be differentially amplified according to their methylation status. Two pairs of primers will be located in the target region; the first pair will complement the target region if all of its CpG-located cytosines remain as cytosines, and the second pair will be complementary if the target region presents uracils in all of its previous CpG sites. The third pair of primers will be designed to complement an area lacking CpG dinucleotides, guaranteeing no possible methylation-dependent allele variation after bisulfite (3). Hence, during the qRT-PCR the first set of primers must amplify the methylated copies of the sequence, while the second set would amplify the unmethylated copies. The third set of primers will amplify DNA without methylation-dependent differentiation, providing a reference of total DNA input as an endogenous control for quantitative assessment of the expression of methylated and unmethylated alleles on the sample by the comparative method (ΔΔCt) (4)

However, the disadvantage of bisulfite-based methods is that they may compromise conversion efficiency due to DNA fragmentation and loss during bisulfite assay, as well as incomplete conversion of unmethylated cytosines (Šestáková et al. 2019). Also, PCR-based methods depend on the correct design of suitable methylated and unmethylated primers, which can be challenging because of the increased tendency of primers to bind multiple sites in bisulfite-treated DNA (Brandes et al. 2007). Nevertheless, today there is a wide variety of commercial kits for bisulfite assay that ensure more than 99% of conversion efficiency, while software specialized in primer design for bisulfite-based methylation analysis protocols help to optimize results. Figure 4 describes some of the main features for feasible methylation-specific primer design.

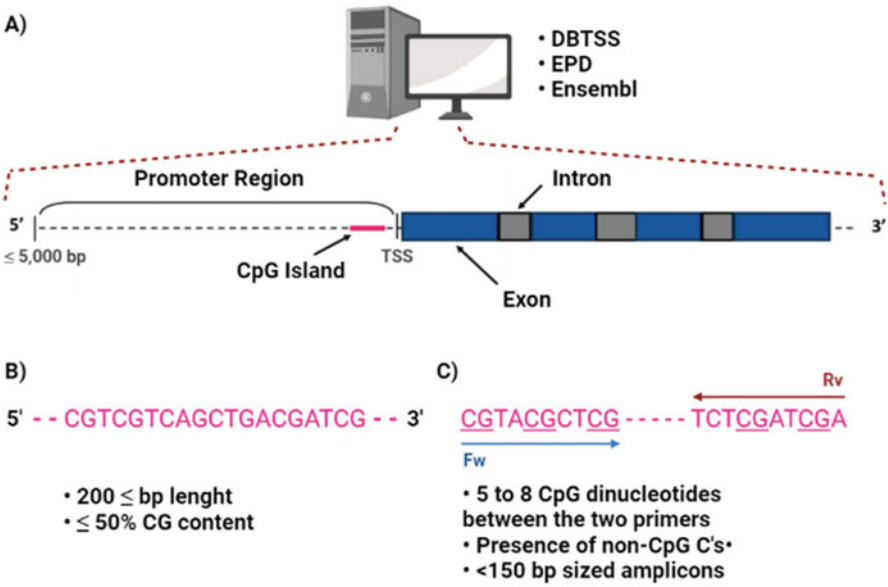

Fig. 4 Main features for optimal primer design for PCR-/qPCR-based gene-specific methylation analysis. (**a**) Gene promoter sequence can be obtained from bioinformatic databases. Some databases like DBTSS (DataBase of Transcriptional Start Sites) or EPD (Eukaryotic Promoter Database) contain reliable sequences for numerous genes of many organisms. If the promoter of interest is not reported, it can be predicted by obtaining the sequence upstream of the transcription start site (TSS). The Ensembl database provides nucleotide sequences for genes distinguishing exons from introns; the promoter region might extend up to 5000 bases upstream the TSS at the beginning of the first exon. (**b**) CpG islands are CG-rich regions of at least 200 bases, most frequently located at gene promoters. The methylation-specific primers must be located at a CpG island as near as possible to TSS but without neglecting the general conditions needed for PCR primer optimization. (**c**) Forward (Fw) and reverse (Rv) primers must be designed for each methylation status. Both primers have to overlap CpG dinucleotides, being 5–8 an optimal between the two. All primers must contain cytosines outside CpG nucleotides to help distinguish bisulfite-treated methylated regions from unconverted DNA. Considering bisulfite-mediated DNA damage, short amplicons (<150 bp) provide best results

Potential DNA Methylation Biomarkers for Transgenerational Neurotoxicity of Arsenic

Global DNA Methylation

The profiling of global DNA methylation has previously been proposed as a biomarker to determine the risk of developing epigenetic alterations due to environmental pollutants such as arsenic (Santoyo et al. 2011). It has been suggested that the joint identification of demethylated cytosines (5hmC) and methylated cytosines (5mC) constitutes a more sensitive biomarker, since 5hmC comes from the oxidation process of 5mC and their levels are correlated. The evidence shows that arsenic influences global DNA methylation and demethylation of cytosines in the brain, as chronic exposure causes a decrease in their levels, specifically in the cortex and hippocampus (Du et al. 2018). Additionally, it has sought to identify the transgenerational effect of arsenic on the global methylation profile in animal models. Nava-Rivera et al. (2021) have reported a multigenerational effect (F0–F3) on cytosine methylation after chronic arsenic exposure in rats, for which they used an ELISA-based assay (MethylFlash, EpiGentek). However, Valles et al. (2020) did not identify changes in the global profile of methylated cytosines in consecutive generations of zebrafish (F0–F1) using the same assay. The inconsistencies could be associated with the technique used, based on a colorimetric test through detection with antibodies that differ from more sensitive techniques such as LC-MS/MS, or a species-dependent effect since there is no sufficient evidence to demonstrate the transgenerational impact of the global DNA methylation profile in zebrafish. Evidence suggests the analysis of 5mC and 5hmC levels as a biomarker of transgenerational toxicity; it has effectively detected alterations after chronic exposure to environmentally relevant doses of arsenic in various organisms and allows for the evaluation of whether the effect is conserved through generations. However, this depends on the applied methodology according to its accessibility and sensitivity.

Differentially Methylated Regions

In addition to analyzing the total methylated cytosines in global DNA, it is possible to detect differentially methylated regions (DMRs). These are genome regions with DNA methylation status differences associated with a specific tissue, a development stage, a specific allele, specific cancer, and/or gene transcription regulation. Importantly, DMRs are frequently located in regulatory regions of transcription, such as gene promoters, since CpG islands are often placed in these sites. Thus, DMRs might be an indicator of alterations in gene expression.

DMRs can be induced by environmental conditions such as exposure to toxic substances. During prenatal and adult stages, arsenic exposures are associated with DMRs containing differentially methylated CpG islands (Bozack et al. 2020; Gliga et al. 2018). Furthermore, it has been suggested that some DMRs can persist after

DNA replication cycles in cell division and embryonic development, despite the reprogramming events which imply methylation erasure (Tuscher and Day 2019).

The above is supported by studies that have identified that DMRs can also originate from ancestral exposure to toxicants. In rodents, exposure to toxic compounds was related to DMR presence in descendants, including up to the F3 generation (Manikkam et al. 2012). Importantly, inherited DMRs were associated with genes involved in different biological processes such as apoptosis, epigenetics, metabolism, signaling, and transcription, as well as a cellular pathway associated with cancer. It suggests that the inheritance of DMRs mediates transgenerational effects of toxic compounds.

Moreover, zebrafish studies have shown that ionizing radiation (an environmental factor widely known for its pathological capacities) is associated with the presence of DMRs, which persist transgenerationally in offspring (F1–F3) and are involved in signaling pathways related to biological processes (Kamstra et al. 2018). Moreover, transgenerational radiation-induced DMRs are primarily located in transcription-regulating sites, such as gene promoters. Therefore, DMRs can act as biomarkers of environmentally induced toxicity and can be analyzed by performing gene-specific methylation analysis.

Gene-Specific Methylation

Since arsenic exposition can alter the expression of specific neural genes through the induction of methylation changes of their promoter, loci-specific methylation analysis is an essential tool to assess arsenic neurotoxicity. The difficulty lies in the fact that many neural genes have been identified as possible biomarkers of methylation-mediated arsenic neurotoxic effects; screening could be performed using a series of general criteria to locate genes in the nervous system that are susceptible to arsenic methylation (1). The gene is conserved in zebrafish (2). The gene is associated with neurodevelopment and brain plasticity and has repercussions on behavior (3). There is evidence that expression is altered, thereby As exposure (4). There are reports of alterations in the methylation of its promoter induced by As or other environmental pollutants. Below are descriptions of some of the primary genes associated with the neurotoxicity of arsenic through methylation changes.

The *Brain-Derived Neurotrophic Factor (BDNF)* and *Tropomyosin-Related Receptor Kinase B (TrkB)* Genes

BDNF protein is a member of the neurotrophin family, which interacts with its receptor, TrkB, to promote neuronal differentiation and survival, playing a role in synaptic plasticity regulation (Moonat and Pandey 2012). The BDNF gene is well conserved in zebrafish, including some regulatory sequences in 5′ compared to mammals (Heinrich and Pagtakhan 2004). The Ntrk2 gene, which encodes for the TrkB receptor, is also conserved in the zebrafish genome (Martin et al. 1995).

Impairment of BDNF-TrkB signaling is associated with neural dysfunction. According to previous studies, BDNF methylation is involved in physiopathological mechanisms of psychiatric diseases, such as schizophrenia and emotional disorders (Ikegame et al. 2013). Altered BDNF-TrkB signaling mediated by epigenetic modifications induced by chemical stressors may be involved in anxiety-like behaviors; in turn, this abnormal behavioral phenotype appears to be transmitted to subsequent generations (Mitchelmore and Gede 2014).

To date, As-exposed human populations (Karim et al. 2019) and experimental models consistently show that the BDNF/TrkB system is the arsenic target; it can alter both gene expression and protein content, while expression can be actively regulated by the methylation of promoter regions (Karpova 2014). Recently, in zebrafish males (Fig. 5), chronic exposure to As produced pronounced hypermethylation of BDNF promoter in the brain (Valles et al. 2020). However, this pattern was not transmitted to other generations, suggesting the complex relationship between phenotypic changes and epigenetic regulation. In the case of BDNF, other epigenetic mechanisms are involved in its regulation, such as H3K4me3 and H3K27ac (Chen and Chen 2017) and

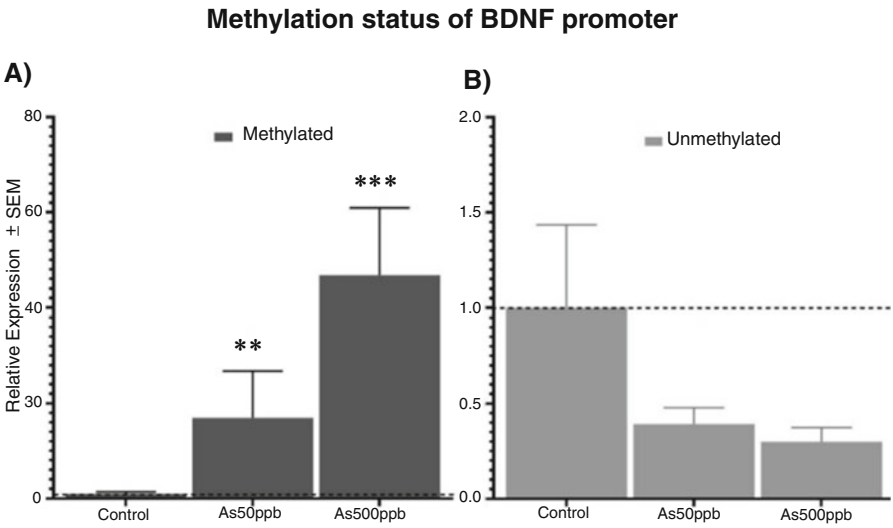

Fig. 5 Analysis of arsenic-induced changes on BDNF promoter methylation through MethySYBR assay. MethySYBR assay shows the abundance of methylated and unmethylated copies of the genomic region of interest, through qRT-PCR which reveals the expression level of both alleles. An increase of the expression of the methylated allele implies there are a larger number of methylated copies, which also applies for the unmethylated allele. In Valles et al. (2020), male zebrafish individuals were divided in three groups according to the arsenic doses they were subjected to (0, 50, and 500 ppb), and MethySYBR analyses were performed to assess the abundance of methylated and unmethylated copies of BDNF promoter in the brain. As result, the individuals showed a dose-dependent increase in the expression of the methylated allele (A), suggesting an arsenic-induced hypermethylation on BDNF promoter. Consistently, the expression of unmethylated allele showed a tendency to decrease (B). For data simplification, the methylation percentage of the target region can also be obtained, by calculating $100/[1 + 2^{(\text{Ct Methylated} - \text{Ct Unmethylated})}]$ for each sample. Resulting data is often easier to visualize in graphs (not added). SEM, standard error of media

small noncoding RNAs (Caputo et al. 2011). Therefore, the literature suggests that BDNF is a critical biomarker to track DNA methylation changes induced by As for single-generation and transgenerational studies.

The *Myelin Basic Protein (MBP)* Gene

MBP is one of the major components of myelin, ranging from 30 to 40% of total myelin protein (Campagnoni 1988). In zebrafish, the MBP gene has low sequence conservation compared to other vertebrates; nevertheless, it performs the same function as in higher vertebrates as the myelin compactor. It has been suggested that arsenic neurotoxicity might be due to the disruption of neural fiber myelination, which negatively impacts neural signal transmission (Zarazúa et al. 2010). In a recent study, rats exposed to arsenic from gestational age had decreased levels of MBP (Niño et al. 2020). Moreover, methylation is widely involved in the synthesis of MBP in the brain, contributing to myelin integrity and degree of compaction (Kim et al. 1997). In rat models, arsenic interferes with the methylation of MBP, thus causing disturbances in nervous transmission through myelinated fibers damage (Zarazúa et al. 2010). This suggests that arsenic could also induce changes in MBP expression through methylation disruption at the DNA level, making MBP a potential DNA methylation biomarker to assess arsenic neurotoxicity.

The *Reelin (RELN)* Gene

Reelin is a protein codified by the RELN gene in humans, conserved in the zebrafish genome. It is widely involved in neuronal plasticity and cognitive function, playing a role in long-term potentiation (LTP). Abnormal reelin expression frequently is seen in various neuropsychiatric disorders, and evidence supports that RELN is regulated by DNA methylation of its promoter. Therefore, the methylation status of the RELN promoter can be an indicator of impaired neural function.

The RELN promoter is susceptible to methylation changes induced by environmental factors such as toxic exposure (Guidotti et al. 2016). Furthermore, previous studies have shown that chronic exposure to arsenic during the early stages of development results in hypermethylation of the RELN promoter, with a negative impact on memory capacities of murine models (Martínez et al. 2011).

The *Protein Phosphatase 1* Gene

Protein phosphatase 1 (PP1) is a multimeric enzyme composed of a catalytic subunit and one or more regulatory subunits. Its catalytic units are conserved in zebrafish, defined as the three isoforms of PP1: PP1α, β, and γ. PP1 is a member of the serine/threonine phosphatases protein group; these enzymes remove phosphate groups from serine and threonine residues in proteins. The dephosphorylation mechanism is key to

neural function since it inactivates many substrates involved in synaptic plasticity. In general terms, PP1 acts as a suppressor of learning and memory, promoting forgetting and thus being a potential mediator of age-related cognitive impairment (Munton et al. 2004). Notably, PP1 expression can be altered by exposure to toxic substances via epigenetic dysregulation. For example, arsenic exposure during gestation results in hypomethylation of the PP1 promoter in the cortex and hippocampus of rats, with a concomitant dose-dependent memory decay (Martínez et al. 2011).

In addition, arsenic has been associated with alterations in the expression of regulatory subunits of PP1, such as PPP1R1B (protein phosphatase 1 regulatory subunit 1B), also known as Darpp-32 (dopamine- and cAMP-regulated neuronal phosphoprotein) (Srivastava et al. 2018). Furthermore, recent studies report alterations in Darpp-32 expression in F1 individuals of zebrafish obtained from an arsenic-exposed F0 generation, showing arsenic can affect the expression of subunits of PP1 intergenerationally (Valles et al. 2020).

The *Cystine/Glutamate Transporter (SLC7A11)* Gene

Cystine/glutamate transporter is a chloride-dependent antiporter that mediates entry of oxidized cysteine into cells in exchange for intracellular glutamate in a 1:1 proportion (Sato et al. 2005). In humans, this antiporter system is X_c^- and encoded by the SLC7A11 gene, conserved across vertebrates including zebrafish (Koppula et al. 2018).

Glutamate is the primary neurotransmitter for excitatory signaling in the central nervous system (CNS). Therefore, dysfunction in the X_c^- system might impair glutamate homeostasis, frequently related to many neural diseases including psychiatric and neurodegenerative disorders (Bridges et al. 2012). Methylation on regulatory sites of the SLC7A11 gene is altered throughout fetal development in humans, consistently with changes in its expression across gestation (Simner et al. 2017). This suggests that the expression of X_c^- system transporters is regulated by DNA methylation.

Recent studies have probed the expression of cystine/glutamate transporters in the cortex and hippocampus of mouse models, noting it is altered by gestational exposure to arsenic and leads to spatial memory impairment (Ramos-Chávez et al. 2015). In addition, arsenic can modify the methylation pattern of SLC7A11 gene in humans (Bozack et al. 2020), suggesting that methylation changes in its encoding gene can mediate arsenic-induced changes in cystine/glutamate transporter expression.

Applications to Prognosis, Other Diseases, or Conditions

The World Health Organization recognizes that at least 140 million people in at least 50 countries use water contaminated with arsenic above the permissible limits (Ravenscroft et al. 2009). The recent emergence of transgenerational studies allows us to explore the generational impact on human health that exposure to As could have. Despite arsenic being one of the most studied substances in toxicology, transgenerational studies are very recent (Table 1). A handful of studies in

Table 1 Studies on the transgenerational effects of arsenic compounds. Summary of the transgenerational studies conducted to date with arsenic. Multigenerational studies were discarded, i.e., the exposure of the organisms occurred directly or as gametes

Arsenic compounds	Arsenic dosage	Toxicological endpoints	Epigenetic biomarkers	Animal model	Number of Generations	Transgenerational effects	Reference
Sodium arsenite	0, 0.2, 1.0, and 5.0 mM	Behavior Gene expression Neurodegeneration	None	*Caenorhabditis elegans*	F1 and F2 (transgeneration)	Altered behavior Increased neurodegeneration	Zhang et al. (2020)
Sodium arsenite	50 and 500 ppb as	Embryotoxicity Behavior Gene expression	Global DNA methylation Gene-specific methylation Histone posttranslational modifications	Zebrafish	F1 and F2 (transgeneration)	Altered behavior Altered histone posttranslational modifications Altered embryotoxicity	Valles et al. (2020)
Arsenic trioxide	1 mg As2O3/mL in drinking water	Reproductive genotoxicity	Global DNA methylation	*Rattus norvegicus* (long-Evans)	F1, F2, and F3 (transgeneration)	Altered reproduction Increased genotoxicity Global hypermethylation	Nava-Rivera et al. (2021)
Sodium arsenite	250 ppb as	Metabolism	None	Mice C57BL/6	F1, F2, and F3 (transgeneration)	Altered glucose metabolism Increased body weight	Gong et al. (2021)

experimental models have demonstrated the transgenerational toxicity of arsenic in different tissues. But the main challenge is to study this phenomenon in human populations to track epigenetic biomarkers associated with exposure and susceptibility to transmission. To date, these studies are virtually nonexistent. In this regard, a promising approach is the multigenerational study (grandparents to grandchildren) conducted with villagers from Hetao Basin, Inner Mongolia, China, historically exposed to doses greater than 500 μg/L As. The study was able to identify a common group of differentially methylated DNA loci and regions (DML/DMRs) associated with an increased risk of skin lesions. Interestingly, some of these DML in patients with skin lesions were shared between grandparents with direct arsenic exposure and their grandchildren with exposure as a gametic cell (Guo et al. 2018). Although this study is not transgenerational, it suggests the possibility of identifying clinically relevant epigenetic biomarkers associated with transgenerational arsenic exposure in humans.

Mini-Dictionary of Terms

DNA methylation	Addition of a methyl group to the DNA molecule that alters its activity without changing the sequence.
Epigenetics	Study of changes in heritable gene expression and activity that are not associated with the nucleotide sequence.
Epigenome	Chemical modifications to DNA and histones proteins that regulate gene expression can be transmitted to the offspring.
Intergenerational epigenetic inheritance	Transmission of epigenetic marks from one generation to the next by direct association with a particular trigger; the exposure starts from the germinal and embryonic stage when the parents are also exposed.
Neurotoxicity	Toxicity caused by natural or artificial toxic agents (neurotoxic) in the central and peripheral nervous systems.
Transgenerational epigenetic inheritance	Transmission of epigenetic marks by the ancestral generation, not attributed to the effects of a particular trigger. It is characterized by having an impact on subsequent generations without having any direct exposure.

Key Facts of DNA Methylation by Arsenic Exposure

- DNA methylation is associated with chromatin organization and consequently with gene expression.

- DNMT catalyzes DNA methylation by transferring a methyl group from SAM to a cytosine, producing 5-methylcytosine.
- Methylation occurs in the cytosines of the CpG dinucleotide. These usually form CpG islands that are present near the promoter regions of genes, so when they are methylated or unmethylated, they can alter gene expression.
- Exposure to arsenic is associated with DNA methylation profile alterations, including both hypermethylation and hypomethylation.
- Modifications of the DNA methylation profile caused by exposure to arsenic can be inherited between generations.
- Arsenic interferes with the DNA methylation process through competition for SAM. It is the methyl group donor for both DNA methylation and arsenic methylation processes which is part of the arsenic metabolism.
- Arsenic interferes with DNA methylation reactions on CpG islands over promoters, which could explain gene expression changes.
- Both long-term and low-dose and acute high-dose arsenic exposure alter DNA methylation patterns.
- Using specific protocols, it is possible to identify global DNA methylation patterns or specific gene methylation.

Summary Points (5–15)

- Arsenic (As) is neurotoxic with the ability to produce epigenetic alterations through generations.
- Arsenic exposure can result in DNA methylation and specific gene methylation.
- The zebrafish model is helpful in evaluating the transgenerational epigenetic effects of arsenic exposure.
- Arsenic exposure alters DNA methylation patterns that are located on numerous genes associated with different biological processes.
- Global DNA methylation, differentially methylated regions, and gene-specific methylation are potential biomarkers to identify transgenerational arsenic toxicity.

Cross-References

► Biomarkers of Neurotoxicity
► DNA Methylation as a Biomarker and Application to Aluminum: *ADRB2* 5′-Untranslated Region (5′-UTR) Methylation Level

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Modeling with Biomarkers: Nitrosamines and Phytochemical Protection

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Abstract

Humans may be exposed to nitrosamines through agricultural pesticides, cosmetics, pharmaceutical preparations, and food preservatives, which are all known to be carcinogenic in the environment. In laboratory animals, nitrosamine chemicals were classed as carcinogenic. The liver is the organ that deals with oxidative stress the most. As a result, prolonged liver damage can lead to major medical problems including fibrosis, which can progress to cirrhosis and liver cancer. Even though the human body can preserve itself within certain limits, the increasing rate of oxidative stress may necessitate the use of external natural antioxidant sources. A large range of natural antioxidants have been shown to have beneficial impacts on human health as well as disease prevention. Specific elements in these plants can also serve as antioxidants; Substances that can either stop or slow down the oxidation process. The purpose of this chapter is to investigate the protective effects of different phytochemicals against the hazardous molecules, nitrosamines.

Keywords

Nitrosamines · Oxidative stress · Phytochemicals · Antioxidants · Medicinal plants · DEN

Introduction

Quite recently, attention has been paid to the impact of environmental pollutants on public health. Among the most dangerous contaminants around us are nitrosamine compounds. They are found in contaminated food including meat or feeding material (representing a high level of risk), drugs, alcoholic beverages, and several vegetables, which represents a threat to living organisms (Veena and Manu 2012). Experimentally, diethylnitrosamine (DEN) is used to stimulate liver carcinoma. DEN metabolism causes elevation of the liver enzymes (e.g., ALT, AST, and ALP) and decrement of antioxidant enzymes in serum of subjected cases due to free radical formation (Atakisi et al. 2013).

The liver is the organ that deals with oxidative stress the most. As a result, prolonged liver damage can lead to major medical problems like fibrosis, which can progress to cirrhosis and hepatocellular carcinoma (Ao et al. 2009). Even though the human body can protect itself within certain limits, the increasing incidence of oxidative stress may necessitate the use of external natural antioxidant sources.

A large range of natural antioxidants have been shown to have beneficial impacts on human health as well as disease prevention (Kelen and Tepe 2008; Hassan and

El-Gharib 2015). Researchers were prompted to focus on analyzing the hepatoprotective effect of various natural antioxidants as a result of the recent surge in demand for healthy meals free of chemicals (Hassan and El-Gharib 2015).

Plants and especially essential oils have been employed as food flavoring and medications in Rome, Greece, and Egypt for thousands of years (Baris et al. 2006). Plants produce primary and secondary metabolites, the latter have different biochemical functions as pollinator attractants and chemical defenses against microorganisms, insects, and predators (Fürstenberg-Hägg et al. 2013). Plants also contain antioxidants in the form of particular components. An antioxidant, according to Matkowski (2008), is a molecule that has the ability to block or postpone oxidation processes. Reactive oxygen species (ROS)-mediated oxidation of fatty acids results in the formation of peroxide, which is then decomposed to produce a variety of secondary products such as aldehydes, ketones, hydrocarbons, and other potentially harmful chemicals for living cells that have been linked to a variety of diseases (Ferrari and Torres 2003).

ROS levels, such as superoxide, have been found to be higher in cancer cells (Ushio-Fukai and Nakamura 2008). As a result of the harmful and/or mutagenic effects of several synthetic antioxidant components, the focus has switched to natural antioxidants. Due to increased uses in the food and cosmetic industries, as well as medical products, the demand for extracts from natural sources such as essential oils is constantly expanding. Hence, additional research is needed to discover the active components in these plants and try to link them to certain functional features such as antioxidant properties in order to uncover potential substitutes for synthetic hazardous preservatives (Sacchetti et al. 2005).

Nitrosamines

Chemical Structure

In 1853, it was found that secondary amines react with nitrous acid or nitrite producing "nitrosoamide," and this was when the nitrosamine compounds were first declared to be discovered (Telling 1982). The resulted product from a combination of an amine or amine derivative with one of nitrosating agents like nitrites, nitrous acid (HONO), is the nitrosamine family, which is also produced from oxidation of nitrogen atom in N-nitroso compounds that contain N-NO group in their chemical structure, such as nitrosamides and nitrosamines.

Sources

Nitrosamine compounds have been found at different matrices such as: foodstuffs (bacon, sausage, cheese, and grain), pharmaceuticals (tetracycline, piperazine, and antihistamines), biological specimens (blood and animal tissues), and environmental specimens (air, water, and soil) (Ikeda and Migliorese 1990).

Cytotoxicity

In experimental animals, nitrosamine compounds are considered one of the carcinogenic compounds, which stimulate cancer formation in the body (Hecht 1981; Pool et al. 1988).

Certain chemical compounds can inhibit nitrosamines formation, such as ascorbic acid, tannic acid, sulfamic acid, α-tocopherol, thiamin, riboflavin, and ammonium sulfamate. These compounds compete with other amines for nitrite species (Ikeda and Migliorese 1990).

Nitrosamines are stable compounds in neutral or strongly basic solutions, but they may be decomposed to nitrous oxide, nitrogen, and aldehydes or to nitrous acid and amines when exposed to ultraviolet UV light spectrum. While, in acidic medium, decomposition occurs slowly except in case of nucleophiles (e.g., chloride and iodide) that increase the rate of reaction (Ikeda and Migliorese 1990).

N-Nitrosodimethylamine (NDMA) and other nitrosamines can be activated into DNA-binding intermediates by the production of -nitrosamino radicals via cytochrome P450 or photochemically. These radical intermediates are either mixed with HO to create hydroxynitrosamines or broken down into nitric oxide and N-methylformaldimine within the catalytic domain of cytochrome P450. Mutagenic -phosphonooxy derivatives are produced in the presence of phosphate from chemically/photochemically generated radicals. Furthermore, in vivo and in vitro studies on lipid peroxidation have indicated that radicals are produced as intermediates from N-nitrosodialkylamines. The extent to which nitrosamine induces lipid peroxidation correlates with its hepatocarcinogenicity in rats. Although preliminary, these findings add to the growing body of evidences indicating the role of free radical damage and DNA alkylation in the carcinogenesis generated by nitrosamines (Bartsch et al. 1989).

The term reactive oxygen species (ROS) is given for both the oxygen free radicals and some non-radical derivatives of O_2 such as hydrogen peroxide (H_2O_2) and organic hydroperoxides (ROOH) (Storz 2005). However, these organic hydroperoxides (ROOH) are not actually free radicals, but they are more likely to be transformed into radicals, and so they are considered as non-radical ROS. Any molecule that has one (or more) unpaired electron(s) is called free radical, e.g., hydroxyl- (•OH), peroxyl- (ROO•), alkoxyl- (RO•), hydrogen peroxide (H_2O_2), and superoxide ($O_2^{\bullet -}$) radicals (Yang 2007).

Many chronic diseases such as cancer are caused due to the presence of ROS, which are widely spread in the body and have both vital and lethal (toxic) effects and may lead to some dangerous diseases. The role of the human body is to eliminate the harmful oxidative effect of these ROS by a powerful antioxidant defense system. On the other hand, this defense system needs to be stimulated due to the decline of its strength in case of aging, so the body should be provided with exogenous antioxidants to maintain its ability to overcome such diseases. The most effective antioxidants were found to be the polyphenols, especially flavonoids (Yang 2007).

Of all free radicals, hydroxyl radical is considered the most pernicious one, because it combines with nearly each molecule in the body cells, including proteins, membrane lipids, and even DNA (Bayir 2005). Hydroxyl radicals are produced from the reactions of iron and/or copper with cell molecules and induce carcinogenesis.

This was proved in laboratory experiments. When rats and mice were injected with ferric nitrilotriacetate, it was found that lipid peroxidation increased, DNA was subjected to oxidative damage, and finally, renal cancer occurred (Toyokuni 1996). Also, the iron-dextran complex, which is used as intramuscular injections for anemia medication in humans, was found to cause many severe problems at its injection location when tested on rats such as pleomorphic and spindle cell sarcoma (Bhasin et al. 2002). Iron is more prevalent in the biological systems; however, the presence of copper ion in some parts of body cells, such as chromatin and DNA bases, especially guanine, makes it very important metal (Kagawa et al. 1991; Wolfe et al. 1994). Copper may cause destruction of DNA when it contributes in Fenton reaction, which leads to the production of site-specific hydroxyl toxic radicals and causes damages to DNA (Wolfe et al. 1994; Hadi et al. 2007).

Despite being very dangerous in their high levels and lead to oxidative stress and toxicity, ROS are very important for some biochemical reactions in the body cells such as intracellular messaging during cell differentiation and cell cycle progression controlling everything from apoptosis (Ghosh and Myers 1998), growth arrest, and immune cell activation (Guangyao et al. 1995; Bae et al. 1997). In other words, the presence of ROS in their normal fluctuating levels is very essential for many body functions, while they turn to be very dangerous when their levels increase over normal level and/or remain constant and can lead to serious damage to lipids, DNA, and protein (Marnett 2000; Stadtman and Levine 2006). Many factors can lead to the dangerous levels of ROS either due to endogenous sources or exogenous sources such as viral/bacterial infection or physical agents (e.g., X-ray, g-ray, and ultraviolet) (Scandalios 2005).

The presence of ROS in low levels is very useful for maintaining the survival of many types of cells (Burdon et al. 1990; Burdon 1995; Burdon et al. 1996; Valko et al. 2006). However, the high and/or constant concentration of ROS can lead to damaging behavior and undesired activities including specific signaling pathways initiation. Also, it can cause genetic modifications which damage DNA and may lead to carcinogenesis (Guyton and Kensler 1993; Cerda and Weitzman 1997). In addition, ROS can stimulate some transcription factors such as the AP-1 family through JNK and p38 MAP kinase cascades (Lo and Cruz 1995; Chung et al. 2002; Storz 2005), interleukin-1 and NF-KB via tumor necrosis factor (TNF) (Baud and Karin 2001; Hughes et al. 2005; Storz 2005). Some critical issues such as apoptosis, carcinogenesis, and cell cycle regulation are caused by the role of ROS in signaling alterations and genetic modifications (Shami et al. 1998; Storz 2005).

The polyunsaturated fatty acid is the major element responsible for keeping the cell membrane fluidity when its side chains exist in the membrane phospholipids. However, free radicals can easily attack these acids leading to the formation of lipid radicals, which react with oxygen on the cell membrane. As a result, peroxyl radicals are produced and continue attacking the proteins of the cell membrane and its polyunsaturated fatty acids, which leads to spreading the peroxidation of membrane lipids (Bayir 2005). This peroxidation produces mainly 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA) (Marnett 1999). The effect of MDA is found to be carcinogenic in rats and mutagenic in mammalian cells (Marnett 1999). While HNE is not very effective as a mutagen, however, signal transduction pathways are

strongly affected by it, and this has a serious impact on the cell phenotypic characteristics (Valko et al. 2006). Moreover, DNA was found to be deeply affected and damaged by lipid peroxidation products such as HNE and MDA (Eder et al. 2006). These peroxidation products do not only damage the DNA but also prevent the essential function of membrane transporters such as the glutamate transporter and Na^+/K^+ ATPases (Hurst et al. 1997; Patterson and Leake 1998).

Phytochemicals

Pharmacologically active substances are richly found and extracted from medicinal plants. Herbs play an important role in various productions, e.g., beverages, flavorings, fragrances, cosmetics, dyeing, charms, nutrition, repellents, smoking, medicine, and industrial uses (Kontogianni et al. 2013). Because of their effectiveness, herbs are prescribed in about 40% of drugs prescriptions (Newman and Cragg 2007).

Definition and Classification of Phytochemicals

Phytochemicals have already been detected in tens of thousands of different plant metabolites, but experts estimate that there is still much more in food that has not been discovered yet. Although the major families of phytochemicals, such as isoflavones, flavonoids, and anthocyanidins, are homogeneous, each category contains unique molecules with unique chemical structures that can be processed differently in the body and may potentially have distinct health impacts (Kallassy 2017). The flavonoids are the most abundant class of phytochemicals. They are a broadly diversified category that has been the most thoroughly examined and chemically recognized. Indeed, over 6000 flavonoids included in plant-based foods for humans have been identified (Tungmunnithum et al. 2018). Fruits and vegetables include a variety of phytochemical substances, including carotenoids, phenolic acids, and ascorbic acid (Liu et al. 2014). Plant polyphenols are found in all diets and sources of wine, tea, vegetables, and fruits; they exhibit substantial antioxidant activity in vitro, which can have a significant impact on clinical therapies and health systems (Skotti et al. 2014). Due to the antioxidant, anti-inflammatory, and antiproliferative properties of phytochemicals, they provide a variety of health benefits (Wang et al. 2020). There is a high association between antioxidant consumption and a decreased chance of developing several diseases such as cardiovascular, cancer, diabetes, and hypertension, as well as other human disorders (Thaipong et al. 2006). Polyphenols and carotenoids are the two main types of antioxidant phytochemicals that confer antioxidant activity on the majority of plants and foods (Stagos 2019). The overall antioxidant activity of phytoconstituents extracts from various plants or meals is directly proportional to their total phenol content. Fruits have a higher antioxidant capacity due to their high level of total phenols (Do et al. 2014).

The category of phytochemicals continues to be a source of contention. Phytochemicals are classified into three categories: polyphenols, terpenoids, and thiols (Ramadan and Ibrahim 2021).

Polyphenols

Polyphenols are the most prevalent phytochemicals in human diet. They are composed of a variety of chemical compounds with a variety of chemical structures, starting from simple monomers to high molecular weight polymers (del Rio et al. 2013). Polyphenols are divided into three categories: flavonoids, phenolic acids, and non-flavonoid polyphenols such as lignans and stilbenes (Kumar et al. 2019). Polyphenols' beneficial effects on health are related to their frequent daily consumption in the diet of humans and their bioavailability. Polyphenols impart color, flavor, bitterness, and astringency to food, as well as maintaining stability by preventing oxidation. Polyphenol consumption has a significant protective effect against a variety of chronic and persistent diseases, including cerebrovascular disease, cardiovascular disease, cancer, ageing, neurodegenerative disease, and diabetes, as demonstrated in numerous epidemiological studies and related meta-analyses (He and Sun 2016).

Terpenoids

Terpenoids, also known as isoprenoids, are a varied collection of naturally occurring compounds derived from terpenes. The majority of them are multi-cycled complexes containing oxygen functional groups. They include terpenoids that are not carotenoid-containing and terpenoids that are carotenoid-containing. The most common type is carotenoids, which are responsible for the red, orange, and yellow colors found in food. Carotenoids, in addition to being antioxidants, protect against some types of cancer, cardiovascular disease, and ciliary, or age-related macular degeneration (AMD) (Meléndez-Martínez et al. 2019).

Thiols

Thiols are chemicals that contain sulfhydryl groups. They are among the most beneficial antioxidants, defending cells against oxidative damage. Glucosinolates, allyl sulfides, and non-sulfur containing indoles are all thiols (Ulrich and Jakob 2019).

Medicinal Plants Role in Prevention of DEN-Induced Cytotoxicity

Capsicum Plant Species

Fruit and resin extracts from *C. annuum* or *C. frutescens* (aka red chilies) were prepared using hexane, ethanol, or vegetable oil to be used safely in a concentration up to 5% in external cosmetics such as skin-conditioning, analgesics, flavoring, and fragrance agents. Among phytocompounds found in *Capsicum* extracts is capsaicin which enhanced the carcinogenic effect of N-nitroso compounds (e.g., N-nitroso

dimethylamine) in mice after oral application. The American Food and Drug Administration (FDA) approved capsaicin to be used as safe and effective external analgesic counterirritant, but not internally, in fever blister and cold sore treatment (Johnson 2007).

Garcinia kola

Seed extract of *Garcinia kola* contains the bioflavonoid kolaviron as a major active ingredient. Kolaviron has several biochemical effects owing to its anti-inflammatory, antioxidant, antigenotoxic properties in addition to its liver protective ability. In an experiment conducted in rats, oral administration of kolaviron (100 and 200 mg/kg) could effectively reduce dimethyl nitrosamine (DMN)-induced hepatotoxicity through lowering levels of blood transaminases and γ-glutamyl transferase and hepatic malonaldehyde as well as restoring normal liver glutathione. Kolaviron hepatoprotective effect was attributed to its ability of downregulating DMN-induced DNA-binding activities of nuclear factor kappa B and activator protein-1, and hence, inhibiting cyclooxygenase and inducible nitric oxide synthase expression (Johnson 2007).

Protium heptaphyllum

The pentacyclic triterpene, β-amyrin, is isolated from *P. heptaphyllum* resin. β-amyrin showed anti-inflammatory, antifibrotic, and antiapoptotic properties in male rats developing dimethyl nitrosamine (DMN)-induced liver fibrosis (Johnson 2007). Application of β-amyrin could effectively normalize all investigated blood markers of liver function, hepatic fibrosis, oxidative stress, tissue inflammatory, and apoptosis. β-Amyrin could then be used as a potential natural solution for controlling the intractable problem of liver fibrosis progression into chronicity which can reduce its consequent high morbidity and mortality rates all over the world.

Cnidoscolus aconitifolius

As demonstrated using gas chromatography and mass spectroscopy techniques, the major phytochemical (45%) in *Cnidoscolus aconitifolius* extracts was found to be the 2,4-bis(1,1-dimethylethyl)-phenol. Extracts also contain carotene, linoleic acid, and other high-silicone constituents. They showed effective medicinal actions against hepatotoxicity, diabetes, and cardiovascular diseases. Moreover, in dimethyl nitrosamine (DMN)-induced toxicity in renal and male reproductive systems in rats, prevention and healing effects of *Cnidoscolus aconitifolius* leaf extract were proved, as compared to ascorbate (Somade et al. 2021). It could normalize levels of all tested kidney function markers (GSH, MDA, GPx, GST, CAT, and SOD) as well as improved DMN-induced abnormalities in sperms in terms of live, concentration, gross, motility, and morphology.

Ipomoea batatas

Anthocyanins isolated from *Ipomoea batatas* (purple sweet potato) are considered as efficient antioxidant and hepatoprotective agents acting through various biochemical pathways. Dimethylnitrosamine (DMN) developed severe liver fibrosis in rats after 4-weeks application characterized by high liver collagen and blood alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (Somade et al. 2021). DMN also leads to loss in both body and liver weights. Prior administration of anthocyanins could prevent all these DMN-induced changes as demonstrated by histopathological analysis and decreased levels α-smooth muscle actin, collagen type I&III, platelet-derived growth factor receptors-β, tumor necrosis factor-α, and transforming growth factor-β. Treatment of rats with anthocyanins could enhance antioxidation which leads to prevention of hepatotoxicity induced by DMN (Somade et al. 2021). This is conducted through nuclear erythroid 2-related factor 2 (Nrf2) signaling pathway by inducing DMN-decreased expression of Nrf2, NADPH:quinine oxidoreductase-1, heme oxygenase-1, and GSTα, while reducing cyclooxygenase-2 and nitric oxide synthase production. Also, anthocyanins reduced inflammation as proved by restoring low serum levels of liver enzymes (ALT, AST, and GST), malonaldehyde, and glutathione, as well as preventing nuclear factor kappa B (NF-κB) translocation.

Zingiber officinale

Ginger (*Zingiber officinale*) is used as a spice and a flavoring agent, with history of medicinal use including arthritis, sore throat, indigestion, dementia, and fever (Liu et al. 2017). It has antioxidant, anti-inflammatory, antihyperglycemic, and anticancer properties (Chrubasik et al. 2005; Shukla and Singh 2007; Ali et al. 2008). Previous studies have concluded that ginger extract has cytotoxic, cellular protective, and chemoprevention effects against cancer (Peng et al. 2012). Among active ingredients of its extracts is zingerone which showed protective activity against liver toxicity and fibrosis caused by dimethylnitrosamine (DMN). Rats with DMN-injured liver were orally treated with zingerone at two different concentrations (10 and 20 mg/kg). Both concentrations could recover induced liver inflammation and fibrosis state caused by DMN through lowering increased content of hydroxyproline and inhibiting liver stellate cells (Somade et al. 2021). Zerumbone (ZER), a mono/sesquiterpene found in subtropical ginger (*Zingiber zerumbet* Smith), exhibits antiproliferative activity against a variety of cancer cell lines, including cervical, cutaneous, and colon cancer. ZER was evaluated for its antitumorigenic properties in rats induced to develop liver cancer using an i.p. injection of diethylnitrosamine (DEN, 200 mg/kg) and feeding on 2-acetylaminofluorene (AAF) (0.02%). ZER preserves the rat liver from the toxic potential of DEN and AAF. ZER-treated rats with liver cancer had decreased levels of ALT, AST, AP, and alpha-fetoprotein. In untreated DEN/AAF rats, MDA levels rose, indicating hepatocellular lipid peroxidation. Liver tissue glutathione concentrations were also significantly reduced. ZER reduced oxidative stress, suppressed proliferation, and promoted mitochondria-regulated apoptosis, reducing DEN/AAF-induced malignancy in rat liver. It thus has considerable potential in treating liver

cancer (Somade et al. 2021). According to the literature, in the DEN-initiated and CCl(4)-promoted hepatocarcinogenesis model in rats, the data revealed that ginger extract may protect against premalignant phases of liver cancer (Mansour et al. 2010). The chemosensitizing impact of *Alpinia officinarum* rhizome extract (AORE) extract in cisplatin-treated hepatocellular cancer rats was also examined. It boosted oxidative stress, hepatic malondialdehyde (MDA), superoxide dismutase (SOD), and catalase activity. Antioxidant gene expression increased in the liver (thioredoxin and glutaredoxin). HCC was identified by histological abnormalities in liver sections and an increase in serum alpha-fetoprotein (AFP). Cisplatin treatment partially improved hepatic functioning and oxidative stress indicators. It also improved histological abnormalities in the liver and AFP levels in serum. It improved liver function and oxidative stress indicators. It also reduced antioxidant gene expression and enhanced liver histopathology and serum AFP levels. This effect is stronger than cisplatin alone. These findings imply that AORE may be a promising natural chemopreventive or chemosensitizing drug (Somade et al. 2021). A recent study reported that the dry ginger and its essential oil succeeded to prevent or reduce the severity of DEN-induced liver injury in rats. Hence, it is recommended to use it as a nutraceutical or dietary supplement (Fahmi et al. 2019).

Litchi chinensis

Processing of *Litchi chinensis* (lychee fruit) extract produced a polyphenol with low molecular weight known as oligonol. Oligonol showed relevant antioxidant, anti-fibrotic, and hepatoprotective properties. After developing of chronic liver injury using dimethylnitrosamine (DMN), rats orally treated with oligonol were almost recovered from hepatotoxicity symptoms (Somade et al. 2021). Oligonol down-regulated transcription of TNF-α, IL-1β&6, cyclooxygenase-2, and inducible nitric oxide synthase. Also, it inhibited hepatic NF-κB, JNK phosphorylation, malonaldehyde, and reactive oxygen species production while restored normal levels of protein SH and nonprotein SH levels. Furthermore, oligonol improved nuclear translocation of Nrf2 and concentrations of phosphorylated Akt and PI3K. Fibrotic markers (including α-smooth muscle actin, transforming growth factor-β1, and type I collagen), collagen content, and hepatic stellate cells activity were recovered.

Stephania tetrandra

The alkaloid tetrandrine (Mwt 622; $C_{38}H_{42}O_8N_2$) could be isolated from *Stephania tetrandra*, a medicinal plant cultivated in China. In addition to its anti-inflammatory effect in lung diseases, tetrandrine also exhibited liver antifibrotic activity both in vitro and in vivo (Somade et al. 2021). After force-feeding with tetrandrine, DMN-induced fibrotic rats showed a significant amelioration in fibrosis scores

(collagen liver content, α-smooth muscle actin, and nuclear factor kappaB). Tetrandrine was able to inhibit the transcription of fibrosis molecular markers (intercellular adhesion molecule-1, α-smooth muscle actin, and transforming growth factor-β1) and decrease serum levels of liver enzymes (ALT and AST).

Tripterygium wilfordii (Hook F)

Mandarin (*Tripterygium wilfordii*), or thunder duke vine, is a Chinese plant used in the traditional medicine from which a diterpene triepoxide called triptolide ($C_{38}H_{42}O_6N_2$) could be isolated. Triptolide was known for its anti-inflammatory and immunosuppressive activities. Antifibrotic effect of triptolide in rats induced by DMN was also proved (Somade et al. 2021). Force-feeding administration of triptolide led to a relevant decrement in all examined scores of hepatofibrosis associated with DMN-hepatotoxicity (including tumor necrosis factor-α, α-smooth muscle actin, nuclear factor-κB, IL-6, and liver collagen content).

Moreover, celastrol is another active phytochemical which could be isolated from *T. wilfordii* and showed anticancer effect in DEN-induced hepatocellular carcinoma in rats (Chang et al. 2016). Celastrol activated apoptosis pathway in liver cells, and hence, decreased morality, normalized hepatic architecture, as well as reduced serum liver markers (ALT, AST, ALP, and AFP) were reported. Celastrol inhibited anti-apoptotic Bcl-2 and Bcl-xl and elevated proapoptotic Bax, cytochrome C, PARP, and caspases. That celastrol reversed DEN-induced HCC in rats may be linked to the death of hepatocellular carcinoma cells caused by celastrol (Chang et al. 2016).

Salvia miltiorrhiza

The Chinese plant *Salvia miltiorrhiza*, or red sage, was known for its relevant use in traditional medicine. Antioxidant activity of red sage extracts did help in protecting rats from oxidative stress associated with DMN-induced liver fibrosis (Hsu et al. 2005). Gavage administration of *S. miltiorrhiza* and silymarin treatment showed comparable fibrotic improvement results, in which a significant decrement was observed in serum AST level, liver collagen content, as well as mRNA levels of procollagen I, α-smooth muscle actin, and transforming growth factor- β1. Furthermore, a mixture of *S. miltiorrhiza, Ligusticum chuanxiong*, and *Glycyrrhiza glabra* also enhanced fibrosis scores including low collagen, α-smooth muscle actin with nuclear translocation factor-κB and decreased mRNA transcription rate of related genes (transforming growth factor-β1, collagen1 α2, α-smooth muscle actin, ICAM-1, and iNOS) (Lin et al. 2008). In the same context, synergistic antifibrotic and anti-inflammatory effect of *S. miltiorrhiza* and *Boswellia serrata*, respectively, against DMN-induced fibrosis was investigated. The plant mixture showed inhibitory effect on transforming growth factor-β1 transcription reflected on ameliorated liver state of fibrotic mice (Sferra et al. 2012).

Polygonum cuspidatum, Morus alba, Vitis vinifera, and Red Wine

P. cuspidatum, M. alba (mulberry), *V. vinifera* (table grapes), and red wine varieties are all considered as rich sources for resveratrol (3,5,40-trihydroxystilbene), a phytoalexin containing polyphenol which has protective effect against several medicinal problems concerning cancer, cardiovascular, nervous, and immune systems. It showed a preventive role in DEN-induced hepatocellular carcinoma in rats (Karabekir and Özgörgülü 2020). Although injection of resveratrol (100 mg/kg) did not result in significant levels of antioxidant markers (malondialdehyde, glutathione, and superoxide dismutase), but a significant decrement in serum levels of liver enzymes (ALT, AST, ALP, and GGT) associated with a significant increment in apoptotic cells was observed. Resveratrol exerts its anticancer effect equally at both early and late stages of hepatocellular carcinogenesis through inducing apoptosis by affecting apoptotic regulators (Rajasekaran et al. 2011). At both cancer stages, resveratrol could recover all DEN-induced changes including liver histology and serum levels of apoptotic markers, hepatocellular carcinoma marker (α-fetoprotein), and liver function markers.

Rosmarinus officinalis

Rosemary (*Rosmarinus officinalis*) is used as flavoring herb with high contents of bioactive compounds. Chemical analysis of its leaves extract showed high content of mono-, di-, and triterpenoids, and phenolic contents, such as carnosic acid and carnosol which have antioxidant and anticancer properties being highly effective in regard to inhibiting cancer cell growth (Bai et al. 2010; Laura et al. 2010; Srancikova et al. 2014; Alanazi 2016; Hanson 2016). The principal phenolic and flavonoid compounds of rosemary powder are hesperidin (4878.88 ppm) and ellagic acid (403.57 ppm). The major active components in essential oil are camphor (18.36%) and α-pinene (12.74%). The preventive efficacy of *Rosmarinus officinalis* (rosemary) powder and essential (volatile) oil against diethylnitrosamine (DEN)-induced kidney damage in rats was examined in a previous study. A considerable increase in blood HDL (28.28%) was observed in rats fed with rosemary essential oil, which was followed by a decrease in LDL (115.47%). There was also a significant drop in serum creatinine and urea (69.72% and 109.89%, respectively). In addition, serum glutathione peroxidase (GSH-Px) activity has increased considerably. The preventive effect against DEN-induced abnormalities was confirmed by histological tests of the kidneys. As a result, rosemary (powder/essential oil) was found to be effective in reducing or even preventing diethylnitrosamine-induced kidney impairment (Hassanen et al. 2020).

Ribes nigrum (Black Currant)

It has been shown that anthocyanins have potent anticarcinogenic activities, indicating a promise for cancer prevention. The anthocyanin concentration in black currant

(*Ribes nigrum* L.) fruits is high. This "superfruit" is known to reduce chronic inflammatory responses. In contrary to the abundance of literature about black currant health advantages, there is scant evidence on its anticancer effects and none on its suppression of experimental carcinogenesis. A BCSE rich in anthocyanins was tested for chemopreventive properties in our well-established rat liver carcinogenesis model. Diethylnitrosamine (DEN) was injected intraperitoneally, followed by phenobarbital to promote hepatocarcinogenesis. Histopathological analysis of liver sections revealed BCSE's antihepatocarcinogenic activity. BCSE inhibited aberrant cell proliferation and promoted apoptosis in DEN-induced rat liver carcinogenesis. The BCSE-mediated proapoptotic signal during experimental hepatocarcinogenesis may be conveyed by translational upregulation of Bax and downregulation of Bcl-2 expression. These findings, together with BCSE safety profile, support its development as a chemopreventive drug for human liver cancer (Bishayee et al. 2011). Further research findings show, for the first time, that reduction of the inflammatory cascade via modification of the NF-B signaling pathway may play a role in the chemopreventive actions of black currant bioactive phytoconstituents against experimental hepatocarcinogenesis, at least in part. These findings, combined with BCSE outstanding safety profile, justify the use of black currant phytochemicals for the prevention or treatment of hepatocellular carcinoma caused by inflammation (Bishayee et al. 2011).

Pomegranate

Pomegranate contains powerful antioxidant and anti-inflammatory compounds. Pomegranate phytochemicals previously inhibited diethylnitrosamine (DEN)-induced hepatocarcinogenesis in mice via Nrf2-mediated antioxidant pathways. Hence, Nrf2 is a crucial mediator in the NF-kappaB-regulated inflammatory cascade. A pomegranate emulsion (PE) was previously studied for its anti-inflammatory properties in rat hepatocarcinogenesis. Induced nitric oxide synthase, 3-nitrotyrosine, heat shock protein 70 and 90, cyclooxygenase-2, and NF-B expression increased in DEN-exposed rat livers. PE reduced the inflammatory markers in a dose-dependent manner. This study found no evidence of PE cardiotoxicity using noninvasive echocardiography to examine heart function. The results showed that modulating the NF-B signaling pathway can decrease the inflammatory cascade, which may be a novel mechanism of PE antihepatocarcinogenic actions. To achieve HCC chemoprevention, pomegranate phytoconstituents must simultaneously target two interrelated molecular circuits, namely Nrf2-mediated redox signaling and NF-B-regulated inflammatory pathway (Bishayee et al. 2011). For the first time, the findings showed that pomegranate components can inhibit hepatocarcinogenesis by upregulating numerous housekeeping genes under the control of Nrf2 without causing toxicity. The findings of this study promote the development of pomegranate-derived products for the management and cure of human HCC, which is still a deadly illness (Bishayee et al. 2011). Furthermore, subsequent findings showed that black currant active anthocyanins have chemopreventive effects against DEN-induced hepatocarcinogenesis by reducing oxidative stress via activation of the Nrf2 signaling pathway (Bishayee et al. 2011).

Grapes, Berries, Peanuts, and Red Wine

The antioxidant resveratrol, found in grapes and red wine, has been shown to reduce diethylnitrosamine (DEN)-induced liver carcinogenesis in rats. Resveratrol is found in grapes, berries, peanuts, and red wine. The amount of nitrite released into the culture medium by activated macrophages was shown to be greatly inhibited by resveratrol, as was the amount of cytosolic inducible nitric oxide synthase (iNOS) protein. Resveratrol reduced the activation of NF kappa B by lipopolysaccharide. Resveratrol reduced both phosphorylation and degradation of nuclear factor inhibitor kappa B alpha (I kappa B alpha). Reactive oxygen species (ROS) are thought to be carcinogenic and linked to tumor growth. Resveratrol may act as a ROS scavenger to inhibit tumor growth. It may also inhibit multistep carcinogenesis by inhibiting mitotic signal transduction. Reactive oxygen species are key players in cardiac disease. There are endogenous antioxidants like SOD, GSHPx, and catalase in tissues. Endogenous antioxidants and reactive oxygen species are thought to be in balance. Increased oxidative stress and subcellular alterations cause cardiomyopathy and heart failure when this balance is disturbed in favor of reactive oxygen species (Bishayee et al. 2011). They also investigated the impact of resveratrol on oxidative stress and inflammatory processes during DENA-induced rat liver carcinogenesis. End-of-study hepatic lipid peroxidation and protein oxidation were significantly higher in carcinogen control animals than in normal controls (20 weeks). The same animals livers had increased expression of inducible nitric oxide synthase and 3-nitrotyrosine. Dietary resveratrol (50–300 mg/kg) reversed all of the above indicators in DEN-exposed mice. HNF-E2-related factor 2 protein and mRNA expression increased by resveratrol (Nrf2). This showed that Nrf2-mediated reduction of oxidative stress and regulation of inflammatory response may be involved in the dietary agent chemopreventive actions against chemically induced hepatic carcinogenesis in rats, and hence recommends the impact of resveratrol in the prevention and treatment of human HCC (Bishayee et al. 2011).

Because cytokines are essential mediators of inflammation, the goal of the previous work was to see how resveratrol affected hepatic cytokines during DEN-induced hepatocarcinogenesis in rats. The effects of DEN on the levels and expression of hepatic TNF-, IL-1, and IL-6 were found to be reversed by resveratrol administration. As a result, resveratrol-mediated prevention and treatment of rat liver carcinogenesis appears to be linked to changes in proinflammatory cytokines (Bishayee et al. 2011). Resveratrol also decreased cell proliferation and induced apoptosis, according to studies. Resveratrol ability to prevent cancer and cardiovascular disease has also been demonstrated (Bishayee et al. 2011).

Rice Bran

Rice bran, particularly that from colored rice, provides a high level of antioxidants. The anticancer properties of hydrophilic purple rice bran extract (PRBE) and white rice bran extract (WRBE) were examined in rats livers following carcinogen-

induced preneoplastic lesion development. Additionally, PRBE inhibited the expression of proinflammatory cytokines including TNF-, iNOS, and NF-B genes. PBRE was shown to possess a higher concentration of anthocyanins and other phenolic components, as well as vitamin E. PRBE may protect rats from DEN-induced hepatocarcinogenesis by reducing cellular inflammation and proliferation. Anthocyanins and other phenolic chemicals, as well as vitamin E, may contribute to rice bran extract's chemopreventive action (Dokkaew et al. 2019).

Garlic and Cinnamon Oils

Natural oils are traditional medicinal plants that have sparked interest because of their anti-inflammatory and anticancer properties. The goal of Zhang et al. study was to see if garlic oil and cinnamon oil may protect rats against p53 gene mutations and hepatocarcinogenesis caused by diethylnitrosamine (DEN) and 2-acetylaminofluorene (2-AAF). Hepatocellular carcinoma was associated with a significant decrease in body mass and a significant increase in liver weight, alpha-fetoprotein (AFP), liver enzymes, hepatic malondialdehyde (MDA), and p53 protein expression levels, as well as genetic mutations in intron 5 of the p53 gene in the form of SNPs and insertions. The levels of glutathione (GSH) and superoxide dismutase (SOD) were also elevated. Although the detrimental effects of garlic oil or cinnamon oil on HCC rats were considerably reversed, the levels of GSH and SOD were elevated. Hypercellularity, nuclear hyperchromasia, mitotic figures, and preneoplastic foci were all found in the HCC-induced group histopathology. HCC rats, on the other hand, showed partial reversal of normal liver architecture after being treatment with garlic or cinnamon oil. The current data suggest that these natural oils can improve liver function while also lowering liver damage and the development of HCC. However, further research is needed before they can be used as standard HCC therapies (Zhang et al. 2012).

Phoenix dactylifera L.

Patients with maintained liver function have a better prognosis and treatment for hepatocellular carcinoma (HCC). Many natural items, including ajwa dates (*Phoenix dactylifera* L.), are reported to have hepatoprotective and HCC-inhibiting properties, although most of them have not been scientifically proven. To test the notion, they used a rat model of diethylnitrosamine (DEN) induced liver cancer to assess the HCC inhibitory effects and other positive features of the aqueous extract of ajwa dates (ADE). The reversion of DEN-damaged liver to normal was aided by ADE. The return to normal levels of antioxidant enzymes, liver enzymes, cytokines balance, and gene expression after ADE treatment suggests that ADE improves liver function and suppresses HCC. As a result, ADE can be administered in conjunction with traditional HCC treatments (Mansour et al. 2010).

Curcuma longa

Curcumin is the active element in *Curcuma longa*, a traditional medication that is widely used. The goal of Kadasa's study was to see if curcumin could protect rats from chemically induced hepatocellular carcinoma (HCC). Fifty albino male rats were separated into five groups (each with 10 rats). The control group received a single intraperitoneal dose of normal saline, the diethylnitrosamine (DEN) group received a single intraperitoneal dose of 200 mg/kg body weight, and the third, fourth, and fifth groups were given DEN and daily administrated curcunine (CUR) via intragastric intubation in doses of 300, 200, and 100 mg/kg b.wt., respectively, for 20 weeks. The levels of alpha-fetoprotein (AFP), interleukin-2 (IL-2), interleukin-6 (IL-6), serum liver enzymes (AST, ALT, ALP, and GGT), as well as the activities and gene expression of glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT), and superoxide dismutase (SOD) were measured in serum and liver samples (SOD). Curcumin reduced serum levels of AFP, IL-2, and IL-6, as well as ALT, ALT, and malondialdehyde (MDA) and IL-2 and IL-6 gene expression. It boosted the gene expression and activity of Gpx, GRD, CAT, and SOD, on the other hand. CUR has been shown to protect albino rats from DEN-induced hepatocarcinogenesis (Kadasa 2015).

Conclusion

The major goal of this chapter is to attract attention to medicinal plants native dietary ingredients, which have a role in preventing diseases caused by reactive oxygen species. This chapter has demonstrated that phenolic and flavonoid components, as phytochemicals, are thought to be responsible for the medicinal plant antioxidant properties. Not only have we looked at the antiradical and antioxidant properties of various plants, but we have also looked at the anticytotoxic properties of some essential oils. In conclusion, it is clear that the antioxidant activity of medicinal plants and their essential oils, as well as their anticancer activities against nitrosamine compound, has been demonstrated in this review chapter. As a result, the antioxidant and anticancer activity potentials of these plants is critical for the future development of new natural antioxidant and anticancer therapies.

Summary Points

- Humans may be exposed to nitrosamines through agricultural pesticides, cosmetics, pharmaceutical preparations, and food preservatives, which are all known to be carcinogenic in the environment.
- In laboratory animals, nitrosamine chemicals are classed as carcinogenic.

- The liver is the organ that deals with oxidative stress the most. As a result, prolonged liver damage can lead to major medical problems like fibrosis, which can progress to cirrhosis and liver cancer.
- Even though the human body can preserve itself within certain limits, the increasing rate of oxidative stress may necessitate the use of external natural antioxidant sources.
- A large range of natural antioxidants have been shown to have beneficial impacts on human health as well as disease prevention. Specific elements in these plants can also serve as antioxidants. It is a substance that can either stop or slow down the oxidation process.
- The purpose of this chapter is to investigate the protective effects of different phytochemicals against the hazardous molecules nitrosamines.

Cross-References

- ▶ Biomarkers of Liver Injury due to Toxic Agents: Progress, Current Applications, and Emerging Directions
- ▶ Erythrocyte Glutathione Transferase P1-1 as a Biomarker in Environmental Toxicology: A New Narrative
- ▶ Modeling with Biomarkers: Nitrosamines and Phytochemical Protection
- ▶ Oxidative Stress Biomarkers and Their Applications to Detect Excessive Fluorine
- ▶ Pro-inflammatory Markers of Environmental Toxicants

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Different Mice Strains in Biomarker Responses to Toxic Agents: The Example of Acetaminophen

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Contents

Abstract

Acetaminophen toxicity is common primarily because the medication is so readily available, and there is a perception that it is very safe. Even though acetaminophen has a good safety profile at therapeutic levels, it can cause severe liver toxicity if taken in large amounts, besides that, acetaminophen toxicity is the

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second most common cause of liver transplantation worldwide. The treatment of acetaminophen poisoning depends on when the drug was ingested and all patients with high levels of acetaminophen need admission and treatment with N-acetylcysteine, an agent that is fully protective against liver toxicity. If the patient is diagnosed and treated promptly, the mortality for acetaminophen toxicity is small; however, if patients present late and have developed severe liver failure, the mortality is increased. In this way it is crucial to increase awareness and understanding of the general population with regards to acetaminophen dosing and toxicity. For that, mice models that mimic the APAP hepatotoxicity observed in humans are essential as study tools for the understanding and treatment of APAP poisoning.

Keywords

Paracetamol · Analgesic · Pain · Xenobiotic · Hepatotoxicity · Biomarkers · Mice strains · Drug metabolism · Mouse models

Abbreviations

APAP	Acetaminophen
AKP	Alkaline phosphatase
AKT	Protein kinase B, also known as *Akt*
ALT	Alanine aminotransferase
AMPK	AMP-activated protein kinase
AST	Aspartate transaminase
BAX	BCL2 associated X
CaMKK	Ca^{2+}/calmodulin-dependent kinase kinase
COX	Cyclooxygenase
COX-2	Cyclooxygenase-2
FDA	Food and Drug Administration
GSH	Glutathione
HO-1	Heme oxygenase-1
ICR	Institute of Cancer Research (strain of albino mice originating in SWISS)
IL-1β	Interleukin-1β
IL-2	Interleukin-2
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
LDH	Lactate dehydrogenase
LKB1	Liver kinase B1
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
NADPH	Nicotinamide adenine dinucleotide phosphate
NAPQI	N-acetyl-p-benzoquinone imine

NPDS	National Poison Data System
NSAIDs	Nonsteroidal anti-inflammatory drugs
NFκB	Nuclear factor-kappa B
Nrf-2	Nuclear factor erythroid-derived 2-like 2
NQO1	NAD(P)H dehydrogenase quinone 1
PCNA	Nuclear antigen proliferating cells
PIC	Poison Information Centre
PI3K	Phosphatidylinositol 3-kinase
PMN	Polymorphonuclear neutrophils
SOD	Superoxide dismutase
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-α
UGT	UDP-glucuronosyltransferase

Introduction

Introduced in the 1950s, acetaminophen (APAP), also known as paracetamol, is one of the most widely used antipyretics and analgesics worldwide, but the mechanism by which acetaminophen produces its analgesic effect is not fully understood (Nakamura et al. 2022). This compound is recognized worldwide as effective for the temporary relief of fever or minor aches and pains (including dental, muscle, headache, menstrual cramps, and minor arthritis pain). Furthermore, APAP is recommended as the first-line remedy for cold, flu, and COVID-19 symptoms (Jaeschke et al. 2021). Since it does not cause gastrointestinal side effects, paracetamol is commonly the first choice for pain management compared to non-steroidal anti-inflammatory drugs (Taouri et al. 2022). This drug is available in a variety of forms including syrup form, regular tablets, effervescent tablets, injection, suppository, and other forms. Moreover, APAP is often found combined with other drugs in more than 600 over the counter allergy medications, cold medications, sleep medications, pain relievers, and other products.

This drug has been determined by health authorities around the world to be safe at recommended daily doses of 4 g/day and less; higher doses may lead to hepatotoxicity and possibly liver failure (Major et al. 2016). However, because of wide availability and minimal cost, acetaminophen is taken in deliberate self-poisoning and unintentional overdose in many countries (Chiew and Buckley 2021), being the most common cause of acute liver failure in the United States, United Kingdom, and Germany (Bernal and Wendon 2013).

Acetaminophen Epidemiology

First reported cases of APAP-induced hepatotoxicity emerged in the United States in the mid-1980s, and since then they have grown. In this country, Paracetamol overdose is responsible for more than 50% of cases of acute liver failure and 20%

of liver transplants, and APAP-induced hepatotoxicity contributes to about 70,000 hospitalizations per year (McGill and Jaeschke 2013; Yoon et al. 2016). The 2020 Annual Report of the American Association of Poison Control Centers' National Poison Data System (NPDS) reported 75,521 case mentions of acetaminophen alone intoxication and 153 deaths among adults and children in the United States (Gummin et al. 2021). Although the majority of overdose cases are related to suicide attempts, the availability of APAP in combination formulations can lead to a chronic and unintentional increase in overdose of this drug (Ramachandran and Jaeschke 2018).

Regarding other countries from the Americas, the Anti-Poison Centre from Québec, Canada, indicated a number of 31,429 APAP-related poisoning cases between 2012 and 2018 (Jiang et al. 2020). In Brazil, 17,031 cases of APAP poisoning, 5666 hospital admissions, and 492 deaths were recorded in the period from 1990 to 2020 (Okuyama et al. 2022). In Colombia, there is no epidemiological data available on APAP intoxication; however, the care of patients with complications from paracetamol overdose in emergency services is frequent (Mancipe et al. 2010). In Chile, medication poisonings correspond to 0.5% of all admissions in emergency services which includes 1197 cases of intentional medication overdose, being APAP responsible for 81 (6.8%) of them from between July 2008 and June 2010 (Aguilera et al. 2016).

A study conducted in Europe analyzed the difference in the proportion of APAP poisoning collected by European's Poisons Information Centre (PIC) with the pack size restrictions of each country. Acetaminophen is frequently used for intentional self-harm in Europe, nevertheless the number of enquiries from 21 PICs indicated no difference between pack size restrictions and intoxication between the years of 2011 and 2013. The United Kingdom, Ireland and Sweden have pack size restrictions and presented a higher percent of APAP-related enquiries of all poisonings, 14.9%, 16.1%, and 4.7%, respectively (Morthorst et al. 2018). In the United Kingdom, APAP is responsible for 50% of poisonings, resulting in 82,000–90,000 hospital admissions and 150–250 deaths per year (as reviewed by Caparrotta et al. 2018). In 2020, 235 deaths were recorded in England and Wales as a result of APAP poisoning (Stewart 2021). The acetaminophen intoxication enquiries in European's PICs, in 2013, were higher than 100 per 100,000 inhabitants in Belgium (609.5), Sweden (606.4), Norway (504.7), Switzerland (296.2), Denmark (173.6), and France (170.6). Slovakia presented the lowest intoxication enquiries per 100,000 inhabitants (0.7), followed by Austria (1), Croatia (7.3), and Italy (10.7) (Morthorst et al. 2018). A study carried out in Spain between 2005 and 2010 found that the incidence of acetaminophen poisoning was 2 cases per 10,000 people in 2005 and 3.4 in 2010, indicating an increasing trend, probably due to the greater formulation, availability of the drug and awareness towards the detection of poisoning cases (Tong et al. 2015).

In Africa, a study conducted in Cape Town, South Africa, recorded daily the poisoning-related hospital admissions in the period between August 2008 and July 2009. The data were collected by the Tygerberg PIC and indicated 136 APAP-poisoning cases in Tygerberg Academic Hospital and 189 APAP-poisoning cases in Tygerberg PIC hospital-based (Veale et al. 2013).

On the Asian continent, according to the 2017 Annual Report of the Israel Poison Information Centre, APAP was the principal analgesic related to poison exposure, with 1352 cases recorded (Bentur et al. 2019). In Hong Kong, the Annual Report of the Poison Information Centre in 2019 described a total of 364 poisoning cases involving APAP (Chow et al. 2021).

In New Zealand, APAP was the most common substance mentioned in the calls to the Poisons Centre, with 309 reports in 2018 (Kumpula et al. 2021). In Australia, the data of annual reports from four PICs (New South Wales, Queensland, Victoria, and Western Australia) indicated that acetaminophen intoxication was also one of the most common reasons for calls to PICs (Chiew et al. 2020). The 2019 annual report collected 5004 case mentions of APAP-related poisoning from 2 Australian PICs (Queensland and Western Australia), among neonates and adults (Queensland Poisons Information Centre Annual Report, 2019; Western Australian Poisons Information Centre: Annual Report 2019).

Since APAP poisoning is a public health concern, we indicate in Table 1 some countries where this problem has been reported. The number of cases presented in Table 1 was normalized per 100,000 inhabitants.

Mechanisms of Acetaminophen Toxicity

Acetaminophen's exact mechanism of action has not been fully established, but despite this, it is often categorized alongside nonsteroidal anti-inflammatory drugs (NSAIDs) due to its ability to inhibit the cyclooxygenase (COX) pathways. It is thought to exert central actions which ultimately lead to the alleviation of pain symptoms (Ghanem et al. 2016).

APAP is often found combined with other drugs in (allergy medications, cold medications, sleep medications, pain relievers, and other products). Confusion about dosing of this drug may be caused by the availability of different formulas, strengths, and dosage instructions for children of different ages. Due to the possibility of fatal overdose and liver failure associated with the incorrect use of APAP, it is important to follow manufacturer dosing guidelines (Yoon et al. 2016).

APAP is the major metabolite of phenacetin and acetanilide. It is mainly metabolized in the liver by first-order kinetics and its metabolism of comprised of three pathways: conjugation with glucuronide, conjugation with sulfate, and oxidation through the cytochrome P450 enzyme pathway, mainly CYP2E1, to produce a reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI). At normal therapeutic doses, NAPQI undergoes fast conjugation with glutathione (GSH) and is subsequently metabolized to produce both cysteine and mercapturic acid conjugates (Mazaleuskaya et al. 2015). The metabolism of APAP by the liver is illustrated in Fig. 1.

Metabolism of APAP can be influenced by polymorphisms in the UDP-glucuronosyltransferase (UGT) enzymes, such as UGT2B15. These variations can result in

Table 1 Worldwide APAP poisoning

Continent	Country	Number of Cases per 100,000 inhabitants	Study Period	Reference
North America	United States of America	22.9	2020	Gummin et al. 2021
	Canada	87.7[a]	2012–2018	Jiang et al. 2020
South America	Brazil	9.2[b]	1990–2020	Okuyama et al. 2022
	Chile	0.5[c*]	2008–2010	Aguilera et al. 2016
Europe	Belgium	609.5	2013	Morthorst et al. 2018
	Sweden	606.4	2013	Morthorst et al. 2018
	Norway	504.7	2013	Morthorst et al. 2018
	Switzerland	296.2	2013	Morthorst et al. 2018
	Denmark	173.6	2013	Morthorst et al. 2018
	France	170.6	2013	Morthorst et al. 2018
	Czech Republic	61.6	2013	Morthorst et al. 2018
	Ireland	59.3	2013	Morthorst et al. 2018
	Lithuania	45.5	2013	Morthorst et al. 2018
	Spain	34.0	2010	Tong et al. 2015
	Finland	14.5	2013	Morthorst et al. 2018
	Italy	10.7	2013	Morthorst et al. 2018
	Croatia	7.3	2013	Morthorst et al. 2018
	Austria	1.0	2013	Morthorst et al. 2018
	United Kingdom	0.9d	2020–2021	Gordon et al. 2021
	Slovakia	0.7	2013	Morthorst et al. 2018
Africa	South Africa (Cape Town)	9.2e	August 2008–July 2009	Veale et al. 2013
Asia	Israel	15.5	2017	Bentur et al. 2019
	Hong Kong	4.8	2019	Chow et al. 2021
Australia	Australia	19.7	2019	Queensland Poisons Information Centre Annual Report 2019; Western Australian Poisons Information Centre: Annual Report 2019
	New Zealand	6.3	2018	Kumpula et al. 2021

[a] 2012–2018 Canadian population average
[b] 1990–2020 Brazilian population average
[c] 2008–2010 Chilean population average
[d] The calculation was made using the number of NPIS consultant referrals between 2020–2021 and using the United Kingdom 2020 population
[e] Data from Tygerberg district, Cape Town (South Africa). The cases per 100,000 inhabitants were calculated using the Cape Town population from 2009
* Intentional medication overdose cases

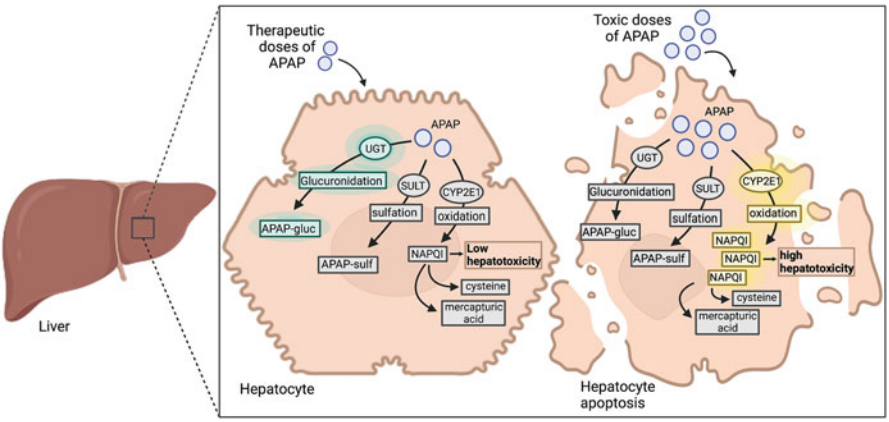

Fig. 1 Metabolism of APAP in the liver at normal and highly toxic doses

higher APAP protein adduct concentrations in some individuals with genetic polymorphisms. APAP protein adducts have been suggested as useful for the diagnosis of an overdose of APAP (Court et al. 2017).

High doses of APAP (overdoses) can lead to hepatic necrosis due to the depletion of glutathione and of binding of high levels of reactive metabolite (NAPQI) to important parts of liver cells. Although rats and mice have been used for more than 40 years in APAP-induced liver injury, rats are much more resistant to APAP hepatotoxicity than mice and they are not a clinically relevant species for studies (McGill et al. 2012b). In this context, murine models are the best choice. Depending on the mouse model of APAP-induced liver injury the doses range from 50 to 600 mg/kg (Tables 2 and 3) resulting in peak necrosis at 12 h in mouse hepatocytes as compared with 48 h in human hepatocytes but the course of liver injury observed in human with APAP overdose are very similar (reviewed by Ramachandran and Jaeschke 2019). The pathophysiological parameters related with APAP-induced liver injury are illustrated in Fig. 1 and the pathological biomarkers evaluated in the mouse models of APAP-induced liver injury are illustrated in Fig. 2.

The liver is the primary organ regulating the nutrition state and energy balance and the second-largest organ and essential for nutrients such as protein, fat, and carbohydrate for their regulation and metabolism (Owumi et al. 2015). Any consumed drug primarily enters the liver before it reaches blood circulation. Since the liver is one of the body's vital organs, its main job is to metabolize and eliminate drugs from the body (Kok-Yong and Lawrence 2015). Hence, the liver is more susceptible to chemical-induced liver injury (Naim et al. 2021).

Pregnant patients, chronic alcoholics, hepatic patients, chronic malnutrition, and severe renal-impaired patients are considered risk groups for developing paracetamol toxicities (Mostafa et al. 2022).

Table 2 Example of alternative approaches for the treatment of hepatotoxicity paracetamol-induced

Hepatotoxicity protocol (dose; route of administration)	Alternative approaches (dose; route of administration)	Parameters evaluated	Mice strain (gender)	Main results	Reference
Plant extracts					
APAP 230 mg/kg; i.p.	*Dendrobium officinale* (50, 100 and 200 mg/kg; oral)	Liver histopathology; Serum levels of ALT and AST; Liver levels of ROS, MDA, GSH, CAT, T-AOC, MPO, Nrf2 activation, HO-1, NQO1	ICR (male)	Reduced hepatic injury; Reduced serum levels of ALT and AST; Reduced ROS, MDA, and MPO and increased GSH, CAT, and T-AOC; Increased activation of Nrf2−Keap1 signaling pathway. Conclusion: Hepatoprotective effect	Lin et al. 2018
APAP 500 mg/kg; i.p.	*Descurainia sophia* (50, 100, 200 and 300 mg/kg; oral)	Liver histopathology; Serum levels of ALT, AST, AKP, total bilirubin, and MDA;	Swiss albino (male)	Reduced hepatic injury; Reduced serum levels of ALT, AST, AKP, total bilirubin, and MDA. Conclusion: Hepatoprotective effect	Moshaie-Nezhad et al. 2018
APAP 200 mg/kg; oral	*Cinnamomum zeylanicum* (200 mg/kg; oral)	Liver and kidney histopathology; Serum levels of ALT, AST, creatinine, urea, of total antioxidant capacity and total oxidant status	BALB/c (gender not specified)	Increased hepatic and renal protective effect; Reduced serum levels of ALT, AST, creatinine, and urea Increased serum antioxidant activity. Conclusion: Liver and Kidney protective effect	Hussain et al. 2019
APAP 250 mg/kg; oral	*Tylophora villosa* (27.5; 55.0; 82.5; 110.0; and 220.0 mg/kg; oral)	Liver aspect (color, weight, volume) Blood glucose;	Swiss Webster (male)	Improved liver aspects and blood glucose; Reduced serum levels of ALT and AST;	Ruyani et al. 2019

		Serum levels of ALT and AST Liver MDA		Reduced liver MDA. Conclusion: Hepatoprotective effect	
APAP 200 mg/kg; oral	*Centella asiatica* (50, 100, and 200 mg/kg; oral)	Liver histopathology; Serum levels of AST, ALT and LDH; Gene expression of proinflammatory cytokines (TNF-α, IL-4 and IL-1β)	BALB/c (male)	Reduced hepatic necrosis; Reduced serum levels of AST, ALT and LDH; Reduced pro inflammatory gene expression. Conclusion: Hepatoprotective effect	Park et al. 2021
APAP 500 mg/kg; i.p.	*Salvadora persica* (500 mg/kg; oral)	Liver and kidney histopathology; Serum levels of ALT, AST AKP, bilirubin, urea, uric acid, and creatinine; Hematological analysis	Swiss albino (male)	Reduced liver and kidney inflammation; Reduced serum levels of ALT, AST, AKP, bilirubin, urea, uric acid, and creatinine; Reduced of hematological changes. Conclusion: Liver and kidney protective effects	Alaraj et al. 2021
APAP 500 mg/kg; oral	*Drynaria quercifolia* (1 and 4 mg/kg; oral)	Liver and kidney histopathology; Serum levels of ALT, AST, AKP, urea, creatinine; Lipid peroxidation level; Liver expression of Nrf2 and activity of SOD1, CAT and GST	Swiss albino (male)	Reduced histological damage in liver and kidney; Reduced serum levels of ALT, AST, AKP, urea, and creatinine; Reduced lipid peroxidation levels; Recover of liver expression of Nrf2, and SOD1, CAT, and GST activities. Conclusion: Liver and kidney protective effects	Chatterjee et al. 2022

(continued)

Table 2 (continued)

Hepatotoxicity protocol (dose; route of administration)	Alternative approaches (dose; route of administration)	Parameters evaluated	Mice strain (gender)	Main results	Reference
Fungi extract					
APAP 400 mg/kg; i.p.	*Sanghuangporus sanghuang* (125, 250 and 500 mg/kg; oral)	Liver histopathology; Liver levels of MDA, CYP2E1, GSH, SOD, GPx and catalase; Liver expression of MAPK, TLR4, PI3K/Akt, NF-κB, Keap1/Nrf2/HO-1 pathway; LKB1/CaMKKβ, and AMPK	ICR (male)	Reduced liver damage, necrosis and degeneration; Reduced liver levels of MDA, CYP2E1 and increased liver levels of GSH, SOD, GPx, and catalase; Reduced liver expression of MAPK, TLR4, PI3K/Akt, NF-κB; Increased liver expression of Keap1/Nrf2/HO-1 pathway; Reduced the phosphorylation of LKB1/CaMKKβ and AMPK expression. Conclusion: Hepatoprotective effect	Jiang et al. 2021
Isolated compounds from plants					
APAP 300 mg/kg; i.p.	Silymarin isolated from *Silybum marianum* (100 mg/kg; oral)	Liver histopathology; Serum levels of AST and ALT; Liver levels of superoxide production (GSSG), HO-1, p-JNK, and *in vitro* mitochondrial superoxide production	BALB/c (male)	Reduced Liver necrosis; Reduced serum levels of ALT and AST; Reduced superoxide production, HO-1, p-JNK, and mitochondrial superoxide production. General: Hepatoprotective effect	Papackova et al. 2018

APAP 250 mg/kg; i.p.	Saponin ginsenoside Rk1 isolated from *Panax ginseng* (10 mg/kg and 20 mg/kg; oral)	Liver histopathology; Serum levels of ALT, AST, TNF-α, IL-1β; Liver levels of GSH, MDA, SOD; Liver expression of cytochrome P450, 4-HNE, 3-NT, iNOS, COX-2, Bcl-2, and Bax proteins	ICR (male)	Reduced hepatic injury; Reduced serum levels of ALT, AST, TNF-α, and IL-1β; Increased levels of GSH and SOD; Increased levels of P450 4-HNE and Bcl-2 levels in liver tissues. Conclusion: Hepatoprotective effect	Hu et al. 2019
APAP 350 mg/kg; i.p.	Dandelion polyphenols isolated from *Taraxacum mongolicum* (100, 200, and 400 mg/kg; oral)	Liver histopathology; Serum levels of AST, ALT, and AKP; Liver levels of SOD; GSH; MDA, TNF-α; IL-1β, iNOS, COX-2, Bcl-2, Bax, caspase-9, p-JNK, Nrf-2 and HO-1	Kunming (male)	Reduced hepatic necrosis, congestion and inflammatory infiltrate; Reduced serum levels of AST, ALT, and AKP; Increased SOD; GSH and reduced MDA Reduced inflammatory factors (TNF-α; IL-1β) Reduced the expression of iNOS and COX-2 and apoptosis (up-regulating Bcl-2 and down-regulating Bax and caspase-9, and up-regulating the expression of Nrf-2) Reduced mitochondrial oxidative stress (down-regulating p-JNK). Conclusion: Hepatoprotective effect	Yong-Shen et al. 2020

Abbreviations: AKP, alkaline phosphatase; ALT, alanine aminotransferase; AMPK, AMP-activated protein kinase; APAP, acetaminophen; AST, aspartate transaminase; CaMKK, Ca^{2+}/calmodulin-dependent kinase kinase; COX-2, cyclooxygenase-2; GSH, Glutathione; HO-1, heme oxygenase-1; Keap1, Kelch-like ECH-associated protein 1; IL-1β, interleukin-1β; IL-2, interleukin-2; iNOS, nitric oxide synthase; LDH (lactate dehydrogenase); LKB1, liver kinase B1; MAPK, mitogen-activated protein kinase; MAPK phosphatidylinositol 3-kinase (PI3K) /protein kinase B (Akt); MDA, malondialdehyde; NFκB, nuclear factor-kappa B; Nrf-2, nuclear factor (erythroid-derived 2) -like 2; SOD, superoxide dismutase; TLR4, Toll-like receptor 4 (TLR4); TNF-α, tumor necrosis factor-α

Table 3 Different mice strains responses to APAP-induced liver injury

Mice strain (gender;age)	APAP concentration and Inoculation route	Main results	Reference
CAST/EiJ, CZECHII/EiJ, C57L/J, NZO/H1LtJ, NOD/LtJ, LP/J, PL/J, WSB/EiJ, BTBR T+ tf/J, SWR/J, A/J, SM/J, WD/PhJ, NON/LtJ, MA/MyJ, RIIIS/J, SJL/J, C57BL/6J, C57BLKS/J, MSM/Ms, PERA/EiJ, SEA/GnJ, AKR/J, FVB/NJ, C57BR/CdJ, 129S1/SvImJ, BALB/cByJ, DBA/2J, JF1/Ms, P/J, C57BL/10J, BUB/BnJ, KK/HlJ, NZW/LacJ, C3H/HeJ, CBA/J (male; 7–9 weeks old)	300 mg/kg; oral	• Mice presented different sensitivity to APAP-induced liver injury; • From 26 genes related with liver necrosis, 16 of them are associated with molecular pathways involved in cell death and proliferation; • While CAST/EiJ mice were more resistant to APAP-induced liver injury, CBA/J mice were more susceptible.	Harrill et al. 2009a
LP/J, C57BL/6J, DBA/2J, NZW/LacJ, and C3H/HeJ[a] (male; 7–9 weeks old)	300 mg/kg; oral	• Polymorphisms in the CD44 gene modulate susceptibility to APAP hepatotoxicity in mice and human samples.	Harrill et al. 2009b
C57BL/6N and C57BL/6J (male; 8–10 weeks old)	200 mg/kg; i.p.	• C57BL/6N mice presented increased formation of APAP-protein adducts and enhancement of mitochondrial dysfunction; • C57BL/6N mice are more susceptible to APAP-induced hepatotoxicity than C57BL/6J.	Duan et al. 2016
C57BL/6 and BALB/c (male; 8–9 weeks old)	50, 250 and 300 mg/kg; i.p.	• Liver mRNA expression TNF-a (pro-inflammatory) was highly induced in C57BL/6 mice, whereas there was no change in BALB/c mice. On the other hand, liver IL-6 (anti-inflammatory) mRNA expression in BALB/c mice was higher than C57BL/6; • C57BL/6N mice are more susceptible to APAP-induced hepatotoxicity than BALB/c.	Masubuchi et al. 2009
C57BL/6 and A/J (male; 8–10 weeks old)	300 or 500 mg/kg; i.p.	• A/J mice presented reduction in circulating	Bavia 2021

(continued)

Table 3 (continued)

Mice strain (gender;age)	APAP concentration and Inoculation route	Main results	Reference
		leukocytes concomitant with the increase in plasma levels of ALT and AST, and massive liver necrosis; • A/J mice are more susceptible than C57BL/6 to APAP-induced hepatotoxicity.	
CD-1 (male; 4–12 weeks old)	600 mg/kg; oral	• Older mice developed more severe hepatotoxicity than younger.	Beierschmitt et al. 1989
ICR (male; 4–12 weeks old)	300 mg/kg; i.p.	• Older mice developed more severe hepatotoxicity than younger.	Taguchi et al. 2015
Korl:ICR, A:ICR and B:ICR (male; age not indicated)	300 mg/kg; i.p.	• APAP-induced hepatotoxicity was the same among mice of different origins.	Jeong et al. 2019
ICR mice and BALB/c	500 mg/kg; i.p.	• APAP-induced liver injury was exhibited earlier in BALB/C than ICR mice.	Muhammad-Azam et al. 2019
C57/B6 and 129/Sv genetic background (male and female; 10–12 weeks old)	500 mg/kg; i.p.	• Female mice were resistant to the hepatotoxic effects of APAP.	Dai et al. 2006
ICR (male and female; age not indicated)	200 and 400 mg/kg; i.p	• Female mice were resistant to the hepatotoxic effects of APAP.	Liang et al. 2013
C57BL/6 (male and female; age not indicated)	150, 300, or 500 mg/kg; i.p.	• Male and female C56BL/6 mice show similar formation of APAP adducts, yet male mice are more sensitive to APAP toxicity.	Mohar et al. 2014

[a]Selected based on the differences in sensitivity to APAP-induced liver necrosis from 36 inbred mice described in the previous line

Treatment for Acetaminophen Overdoses: Classic and New Approaches

Before an effective antidote was developed, the morbidity following APAP overdose was high. The abovementioned damage to the liver can be prevented by the early administration of sulfhydryl compounds, for example, methionine and N-acetylcysteine. Acetylcysteine is by far the most widely used antidote for APAP toxicity (Bateman and Dear 2019).

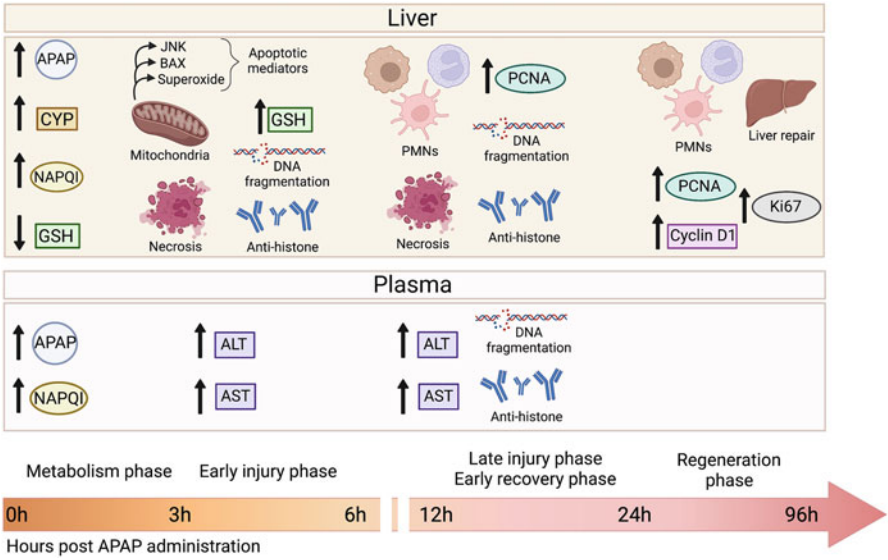

Fig. 2 Pathological Biomarkers of APAP in the mouse model

Acetylcysteine is a precursor that is hydrolyzed intracellularly to cysteine, which is the rate-limiting factor in replenishing glutathione and thus it stimulates glutathione synthesis. Acetylcysteine also supplies thiol groups, which can directly bind with NAPQI in hepatocytes and enhances nontoxic sulfate conjugation. The efficacy of oral and intravenous acetylcysteine is similar (Chiew and Buckley 2021).

Although acetylcysteine is the treatment of choice, adverse effects such as nausea, emesis, and anaphylactoid reactions have been reported depending on protocol of administration (Hoffman 2020). On the other hand, plant (Lin et al. 2018; Moshaie-Nezhad et al. 2018; Hussain et al. 2019; Ruyani et al. 2019; Alaraj et al. 2021; Park et al. 2021; Chatterjee et al. 2022) or their purified components (Papackova et al. 2018; Hu et al. 2019; Yong-Shen et al. 2020), as well as fungi extracts (Jiang et al. 2021) have been studied as an alternative treatment option. There is much data in experimental animal models demonstrating their ability to act as a restorer of the defense and antioxidant system (Subramanya et al. 2018). In these studies, the doses of APAP employed in these studies range from 200 to 500 mg/kg and the main route of administration is intraperitoneal followed by the oral (gavage). In addition, the main mice strains used are ICR, Swiss albino, and BALB/c. Furthermore, the main effects and pathological biomarkers investigated include: histopathological evaluation, liver integrity biomarkers [aspartate transaminase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AKP)], lipid peroxidation [malondialdehyde (MDA)], antioxidant and oxidative stress [glutathione (GSH), superoxide dismutase (SOD), catalase, nuclear factor erythroid-derived 2-like 2 (Nrf2) activation, heme oxygenase-1 (HO-1)], mitochondrial superoxide production, and pro-inflammatory cytokines, just to mention a few.

Thus, alternative approaches employing plant extracts for the treatment of APAP overdoses have been shown promise (Table 2).

Mice Models of Acetaminophen Toxicity

Mice are very adequate as experimental animals for understanding metabolic functions, pathogenic mechanisms, and immune manifestations in APAP overdose. Due to the availability of immunological markers and antibodies for mice, the easy handling, and the availability of knockout animals, this species is very suitable for studying hepatotoxicity mechanisms (Mossanen and Tacke 2015). Thus, mice models that mimic the pathophysiology of APAP hepatotoxicity observed in humans will also contribute to understanding of poison, as well as, improve the treatment of overdose.

Routes of Inoculation of APAP

In clinical practice, APAP is used both orally and intravenously. Oral APAP is known to have a rapid absorption with a systemic bioavailability ranging from 70 to 90%. Despite these favorable parameters, in cases with impaired consciousness, nausea, vomiting, and gastrointestinal problems impeding gastrointestinal absorption, intravenous (IV) administration of APAP is preferred (Asci et al. 2022).

In the experimental model, the administration of APAP can be via intraperitoneal, intravenous injection, or oral by gavage, with each offering advantages and disadvantages depending on the specific goal of the study (Fig. 3). A period of fasting is necessary so that the conditions for metabolizing APAP are comparable (indicated in the literature is between 12 and 16 h with free access to water). The intraperitoneal route is the most used, due to its high reproducibility and ease of handling, but can lead to local irritation of the skin. Intravenous administration is performed via injection into the lateral tail vein and has even higher reproducibility. Usual doses of APAP for intraperitoneal injection range from 250 mg/kg to 500 mg/kg body weight into the lower side of the abdomen. The usual dose for the intravenous route is 250 mg/kg of body weight, after a 12 h fasting period (Mossanen and Tacke 2015).

Administration by gavage is not recommended for the study of APAP hepatotoxicity, as it presents great variability in absorption due to individual differences needing to increase the number of experimental animals (Mossanen and Tacke 2015). The maximum volume for rodents is 1 mL of solution for every 100 g of body weight. Due to its low acidity and lipid solubility, APAP is rapidly absorbed from the intestine after an oral dose is administered (McGill et al. 2012a).

Animals submitted to the liver injury model do not show signs of pain, as do humans. This is believed to occur because APAP acts as an analgesic. The signs observed in the model are fatigue and delayed locomotion after 12 h of injection of APAP (Mossanen and Tacke 2015).

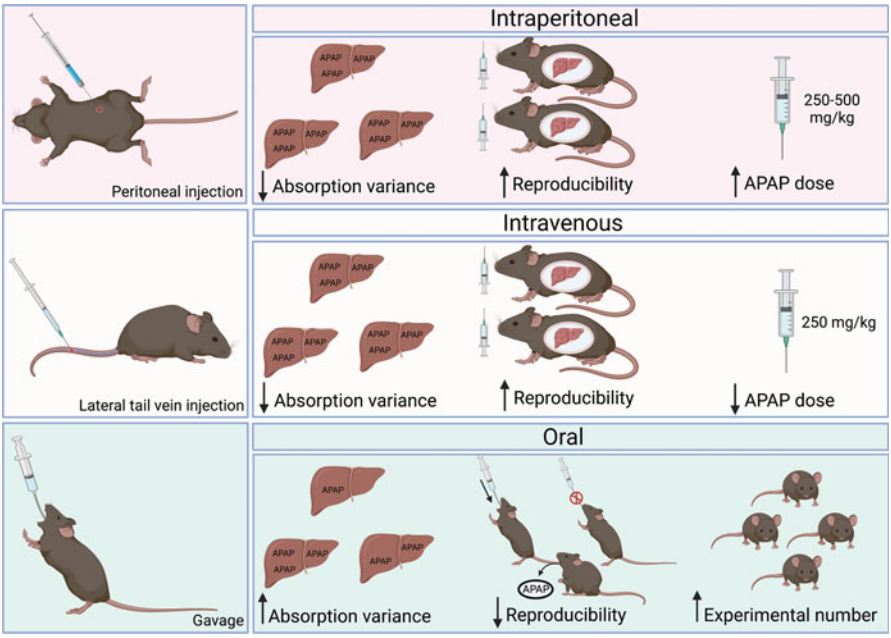

Fig. 3 Routes of administration in C57BL/6 model of APAP-induced hepatotoxicity

Different Mice Strains Responses to Acetaminophen

The basic mechanisms of APAP hepatotoxicity are shared by several strains of mice; however, genetic differences modulate the induced damage (Jaeschke et al. 2021). In order to highlight response biomarkers for hepatotoxicity in genetically diverse populations, mice from 36 inbred strains were treated with an intragastric dose of 300 mg/kg of APAP (Harrill et al. 2009a). Based on the percentage of liver necrosis, these 36 inbred mice were ranked regarding their sensitivity to APAP-induced liver injury: 0–10% of liver necrosis (CAST/EiJ, CZECHII/EiJ, C57L/J, NZO/H1LtJ, NOD/LtJ, LP/J), 10–20% of liver necrosis (PL/J, WSB/EiJ, BTBR T+ tf/J, SWR/J, A/J, SM/J, WD/PhJ, NON/LtJ, MA/MyJ, RIIIS/J), 20–30% of liver necrosis (SJL/J, C57BL/6 J, C57BLKS/J, MSM/Ms., PERA/EiJ, SEA/GnJ, AKR/J, FVB/NJ, C57BR/CdJ, 129S1/SvImJ), 30–60% of liver necrosis (BALB/cByJ, DBA/2 J, JF1/Ms., P/J, C57BL/10 J, BUB/BnJ, KK/HlJ, NZW/LacJ, C3H/HeJ), and, 60–100% of liver necrosis (CBA/J). Furthermore, the analysis of liver gene expression data revealed 26 genes that correlated with liver necrosis, which when evaluated by Ingenuity Pathway Analysis reveal that 16 genes of the biomarkers are closely associated with molecular pathways involved in cell death and proliferation (Harrill et al. 2009a).

In a similar study, employing the same 36 inbred mice strains, differences in metabolism of APAP were investigated interstrain, and five them (LP/J, C57BL/6 J, DBA/2 J, NZW/LacJ, and C3H/HeJ) were selected based on the differences in sensitivity to APAP-induced liver necrosis (Harrill et al. 2009b). In this study

whole-genome association analysis and targeted sequencing were applied to evaluate polymorphisms in *Ly86*, *Cd44*, *Cd59a*, and *Capn8* genes. The authors found a strong correlation between these polymorphism and liver injury, and demonstrated that dose-curves vary with mice genetic background. Furthermore, variation in the orthologous human gene, *CD44*, that codes a cell-surface glycoprotein important to cellular interactions, adhesion, and migration (Senbanjo and Chellaiah 2017) was associated with susceptibility to acetaminophen indicating that CD44 may modulate susceptibility to APAP hepatotoxicity in both humans and mice (Harrill et al. 2009b).

The C57BL/6 mouse strain is widely used in APAP-induced liver injury because it is the background strain of mice with knock-out genes. Even so, there are two C57BL/6 substrains, 6 N and 6 J, which present differential susceptibility to APAP-induced liver injury. C57BL/6 N mice showed more liver, higher glutathione disulfide-to-glutathione ratio, and increased mitochondrial dysfunction after APAP exposure (Duan et al. 2016).

Comparing C57BL/6 mice with BALB/c, strains with different Th1 and Th2 immunological response, C57BL/6 mice show higher expression of tumor necrosis factor alpha (TNF-a) and greater liver damage compared to BALB/c mice. The authors suggest that this response to APAP exposure may be due to C57BL/6 mice presenting a specific Th1-dominant response (Masubuchi et al. 2009). On the other hand, A/J mice that present a Th2 immune response in some models, are more susceptible than C57BL/6 mice when treated with APAP (Bavia 2021). Probably, these differences among liver damage in each mice strain may be related to the amounts of CYP2E1 protein in the liver, which is known to be 40% higher in A/J than in C57BL/6, followed by CD-1 mice (Forkert and Boyd 2001). It is interesting to mention that A/J mice, as well as SWR/J, AKR/J, DBA/2 J mice strains employed by (Harrill et al. 2009a, 2009b) are genetically C5 deficient (Wetsel et al. 1990), an important component of the complement system.

Regarding CD-1 mice strain, it is noteworthy that young animals are more susceptible to APAP-induced acute liver injury when compared to adult animals (Beierschmitt et al. 1989). In consonance with these findings, age-related changes in susceptibility to APAP-induced liver injury were also observed in ICR mice strain (Taguchi et al. 2015) (Taguchi et al. 2015). In addition, the APAP-induced liver injury is equivalent in ICR mice strains from different sources (Korea, United States, and Japan) (Jeong et al. 2019).

Comparing APAP toxicity between ICR and BALB/c mice, intense centrilobular damage was observed early in BALB/C than in ICR mice. The author suggests that this is due to differences in the genetic background of each strain involved with the APAP metabolism (Muhammad-Azam et al. 2019).

The differences between female and male mice in APAP-induced liver injury are well established. While males are highly susceptible, females are strongly resistant to the hepatic toxic effects of APAP overdose. These differences are independent of genetic background (Dai et al. 2006; Liang et al. 2013; Mohar et al. 2014). However, for female APAP-induced liver injury models, a dose adjustment may be necessary (Mossanen and Tacke 2015).

In summary, mice from different genetic strains can be used in the hepatotoxicity model, and the APAP dose must be adjusted according to the strain and severity of the lesions in the model.

Applications and Importance of APAP Studies

*The great occurrence of APAP poisoning worldwide reinforces the importance of APAP studies, mainly these one that investigate biomarkers of APAP-induced hepatotoxicity. Although the number of studies investigating the pathophysiology triggered by overdose in paracetamol consumption is large, the lack of data is still a concern (*Zyoud et al. 2015*). Mice models that mimic the APAP hepatotoxicity observed in humans are of great value as study tools and as models for the development of new approaches to treatment of APAP poisoning (*Jaeschke et al. 2014*). Thus, studies about APAP poisoning are still an important issue in scientific research and require attention regarding the toxicological aspects and the identification of biomarkers in response to APAP toxicity.*

Mini-Dictionary of Terms

- ***Analgesic****. Also called pain reliever or painkiller, is a* drug *used to achieve relief from pain.*
- ***Overdose****. Set of effects caused by excessive consumption of drugs or medication.*
- ***Hepatotoxicity.*** *Toxic liver disease.*
- ***Xenobiotics****. Chemical substances not normally present in the environment of living organisms.*
- ***Sulfhydryl compounds.*** Cytoprotective *agents that prevent the acute hemorrhagic erosions caused by* anti-inflammatory *drugs.*
- ***Intraperitoneal route****. Injection where a pharmacological agent is injected into the peritoneal cavity.*
- ***Gavage.*** *Introduction of substances into the stomach using a tube or a cannula.*
- ***Hepatic necrosis.*** *Death of liver cells.*
- ***Epidemiology.*** *Study of health-related events in specified populations.*
- ***Strains of cells.*** *Cell culture that contains only one type of cell and has a finite capacity to replicate.*
- ***Inbred mice strain.*** *Mouse strains that have been maintained by successive brother to sister matings over more than 20 generations.*
- ***Mouse model.*** *Laboratory mice that are used to study some aspect of human physiology or disease.*

Key Facts of Acetaminophen Toxicity

- *Pharmaceuticals play a pivotal role in our society, which commonly uses, and often, a number of these molecules.*

- *Acetaminophen is one of the most widely used antipyretics and analgesics worldwide.*

Abbreviations Such as APAP, AC, Acetaminop, Acetamin, or Acetam, May Be Written on the Label in Place of the Word Acetaminophen

- *People can accidentally take too much acetaminophen, if they do not follow the directions on the prescription or package label carefully.*
- *Paracetamol can be hepatotoxic to overdose, a condition documented in both experimental animals and humans.*
- *Paracetamol-induced hepatotoxicity typically occurs when concentrations of the metabolite NAPQI reach toxic thresholds.*
- *Acetaminophen overdose needs admission and treatment with N-acetyl-cysteine,* administered orally or intravenously.

Summary Points

- *The perception of acetaminophen as a safe drug has become very misleading as this has led to a high rate of paracetamol toxicity.*
- *When paracetamol is taken in excessive quantities of 4 g/ daily, it leads to serious negative effects for human health.*
- *Liver failure (hepatic necrosis) is reported even with doses just more than the maximum therapeutic dose.*
- *Acetaminophen metabolism primarily occurs through glucuronidation and sulfuration in the liver. In an overdose, these pathways are saturated, and more acetaminophen is subsequently metabolized to NAPQI by cytochrome P450.*
- *Plant products, extracts, or purified components have been considered for treatment options in acetaminophen intoxications.*
- *Mouse models of acetaminophen hepatotoxicity are considered relevant for human and animal pathophysiology.*
- *Several mice strains share the basic mechanisms of APAP-induced hepatotoxicity; however, genetic background modulates the damage magnitude.*

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Part VII

Resources

Recommended Resources for Biomarkers in Disease: Toxicology

50

Rajkumar Rajendram, Daniel Gyamfi, Vinood B. Patel, and Victor R. Preedy

Contents

Abstract

It is extremely challenging to assess patients exposed to toxins. An accurate history is difficult to obtain; thus, tools and platforms for toxicological analysis are required. Biomarkers can be objectively measured and evaluated and are extremely useful in toxicology. They can be used to indicate susceptibility, exposure, and effect. This can facilitate risk assessment and treatment. Thus,

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biomarkers can prevent and reduce the harmful effects of select toxins. Some biomarkers can also provide great insight into the underlying pathophysiological mechanisms of toxicity. The application of biomarkers is actively being investigated for a variety of toxins, and the understanding of this topic continues to be advanced. However, keeping abreast of current research is difficult so we have compiled resources recommended by active toxicologists, practitioners, and researchers. These include information on regulatory bodies, societies, organizations, and other resources.

Keywords

Books · Evidence · Journals · Development · Professional societies · Regulatory bodies

Introduction

The clinical assessment of patients exposed to toxins can be extremely challenging. Obtaining an accurate history is difficult as patients may be unable or unwilling to provide a clear account of the events leading to the exposure. Those at greatest risk from the complications of toxins (e.g., alcohol misusers: Rajendram and Preedy 2008) are particularly unlikely to be cooperative or even aware of the exposure and/or the associated risks. Thus, accurate tools for toxicological investigations are required.

Biomarkers are characteristics that are objectively measured and evaluated (Atkinson et al. 2001). They can be used to demonstrate physiology, risk, clinical trajectories, pathophysiology, or responses to therapies (Atkinson et al. 2001; Rajendram et al. 2023). Biomarkers are, therefore, extremely useful in toxicology.

In the acute setting, several biomarkers are required to provide as complete a picture as possible in patients who have been exposed to a toxin and are, or may become, critically ill. However, there is no clear consensus on the best biomarkers for the field of toxicology. Rather, in toxicology biomarkers may be generally classified as biomarkers of susceptibility, exposure, and effect (Aitio et al. 1993). Biomarkers of susceptibility can predict patients' response to toxins (Aitio et al. 1993; Nordberg 2010). Biomarkers of exposure are early indicators of potential toxicity (Aitio et al. 1993). They arise after a chemical agent interacts with its target molecule (Aitio et al. 1993; Nordberg 2010). In conjunction with biomarkers of early disease detection, biomarkers of exposure facilitate the development of personalized strategies to treat toxicological disease. Effect biomarkers are late markers. They measure damage to target organs and patients' overall health, after exposure to a toxin (Aitio et al. 1993).

In toxicology, biomarkers are clinically useful tools that facilitate risk assessment and treatment. Thus, biomarkers can prevent and reduce the harmful effects of selected toxins. Some biomarkers can also provide great insights into the underlying pathophysiological mechanisms of toxicity. Such mechanistic biomarkers are often of greatest benefit in clinical decision-making. However, no single toxicological

biomarker is applicable to all situations. So, several biomarkers may be used in particular circumstances and may range from indicators of exposure to end organ damage.

Mechanistic biomarkers can provide data relevant to exposure, effect, and susceptibility (Robinson et al. 2013). Mechanistic biomarkers can provide prognostic data. Yet, these biomarkers are not used for follow-up or the assessment of response to treatment (Robinson et al. 2013). Genetic trait indices are among the best mechanistic biomarkers. However, several biochemical processes, including oxidative stress, organelle damage, and biotransformation, can also generate detectable biomarkers.

Since Aitio et al. (1993), the science and application of biomarkers has been actively investigated for a variety of toxins. More lately, these include, as examples, ethanol (Tawiah et al. 2022), lead (Salcedo-Bellido et al. 2021), aluminum (Salcedo-Bellido et al. 2021), mercury (Salcedo-Bellido et al. 2021), radiological contrast agents (D'Amore et al. 2020), and numerous other areas covered in Patel et al. (2024).

In this chapter, we have produced tables containing resources as recommended by active researchers and practitioners. The information contained therein draws upon the knowledge, experiences, *know-how,* and acumen that has been acquired over years of research. The list below acknowledges all the experts who helped to prepare these resources.

Resources

Tables 1, 2, 3, 4, and 5 list the most up-to-date information on the regulatory bodies (Table 1), professional societies (Table 2), books (Table 3), emerging technologies, and platforms (Table 4), and other resources of interest (Table 5) that are relevant to an evidence-based approach to biomarkers of toxicology. Some organizations are listed in more than one table as they occasionally fulfill multiple roles.

Other Resources

The Wellcome Collection (https://wellcomecollection.org/collections) and The British Library (https://www.bl.uk/) are important sources of biomedical information including toxicology and biomarkers.

Other chapters on resources relevant to biomarkers (recommended by authors and practitioners) may also be relevant to biomarkers of toxicology. These include nutrition and oxidative stress (Rajendram et al. 2020), general aspects of biomarkers (Rajendram et al. 2016a), biomarkers of cardiovascular disease (Rajendram et al. 2016b), biomarkers of renal disease (Rajendram et al. 2017), and aging (Rajendram et al. 2021).

This list of material in these tables is included to provide general information only. It does not constitute any recommendation or endorsement of the activities of these sites, facilities, or other resources listed in this chapter, by the authors or editors of this book.

Table 1 Regulatory bodies or organizations dealing with biomarkers or toxicology. This table lists the regulatory bodies and organizations involved with biomarkers or toxicology. The links were accurate at the time of going to press but may move or alter. In these cases, the use of the "Search" tabs should be explored at the parent address or site. In some cases, links direct the reader to pages related to biomarkers of toxicology within parent sites. Some societies and organizations have a preference for shortened terms, such as acronyms and abbreviations. See also Table 2

Regulatory body or organization	Web address
Academy of Toxicological Sciences	http://www.acadtoxsci.org/
Agency for Toxic Substances and Disease Registry (ATSDR)	https://www.atsdr.cdc.gov/
American Academy of Clinical Toxicology	http://www.clintox.org/
American Board of Forensic Toxicology (ABFT)	http://www.abft.org/
American Board of Toxicology (ABT)	http://www.abtox.org/
American Board of Veterinary Toxicology (ABVT)	https://www.abvt.org/
American College of Cardiology	https://www.acc.org/
American College of Toxicology	http://www.actox.org/
California Department of Food and Agriculture (CDFA)	https://www.cdfa.ca.gov/
Centers for Disease Control and Prevention (CDC)	https://www.cdc.gov/
Coalition to Prevent Lead Poisoning (CPLP)	https://theleadcoalition.org/
European Medicines Agency (EMA)	https://www.ema.europa.eu/en
Health and Environmental Sciences Institute (HESI)	http://www.hesiglobal.org/
Human Proteome Organization	https://www.hupo.org/
International Agency for Research on Cancer (IARC), WHO	https://www.iarc.fr
International Union of Toxicology (IUTOX)	http://www.iutox.org/
Mayo Clinic	https://www.mayoclinic.org/
National Health Portal of India (NHP India)	http://nhp.gov.in/
National Health Surveillance Agency (ANVISA)	https://www.gov.br/anvisa/pt-br
National Institute of Aging (NIA)	https://www.nia.nih.gov/
National Institute of Environmental Health Sciences	https://www.niehs.nih.gov/
Organisation for the Prohibition of Chemical Weapons (OPCW)	https://www.opcw.org/
Pan American Health Organization (PAHO)	https://www.paho.org/en
Toxicology Education Foundation (TEF)	https://toxedfoundation.org/
U.S. Environmental Protection Agency (EPA)	https://www.epa.gov/
U.S. Food and Drug Administration	https://www.fda.gov/
U.S. National Office for Harmful Algal Blooms (HABs)	https://hab.whoi.edu/
United Nations Educational, Scientific and Cultural Organization (UNESCO)	https://www.unesco.org/en
United States Department of Agriculture (USDA)	https://www.usda.gov/
World Bank Group	https://www.worldbank.org/en/home
World Health Organization (WHO)	https://www.who.int/

Table 2 Professional societies relevant to biomarkers or toxicology. This table lists the professional societies involved with biomarkers or toxicology. The links were accurate at the time of going to press but may move or alter. In these cases, the use of the "Search" tabs should be explored at the parent address or site. In some cases, links direct the reader to pages related to biomarkers of toxicology within parent sites. Some societies and organizations have a preference for shortened terms, such as acronyms and abbreviations. See also Table 1

Society name	Web address
American Association for Cancer Research	http://www.aacr.org/
American Association for Clinical Chemistry	https://www.aacc.org/
American Association for Laboratory Animal Science	http://www.aalas.org/
American Chemical Society	https://www.acs.org/
American Chemical Society Division of Toxicology	http://www.acschemtox.org/
American Fisheries Society	https://fisheries.org/
American Public Health Association (APHA)	https://apha.org/
American Society for Mass Spectrometry	https://www.asms.org/
American Society of Safety Professionals	https://www.assp.org/
British Toxicology Society	https://www.thebts.org/
European Society of Toxicologic Pathology (ESTP)	http://www.eurotoxpath.org/
European Society of Toxicology	https://www.eurotox.com/
International Association of Therapeutic Drug Monitoring and Clinical Toxicology	http://www.iatdmct.org/
International Society for Environmental Epidemiology (ISEE)	https://www.iseepi.org/
International Society for the Study of Xenobiotics	http://www.issx.org/
International Society of Mycotoxicology (ISM)	www.mycotox-society.org
Society for Risk Analysis (SRA)	http://www.sra.org/
Society of Toxicologic Pathology (STP)	http://www.toxpath.org/
Society of Toxicology (SOT)	http://www.toxicology.org/

Summary Points

Toxicological biomarkers are of great clinical significance.

Toxicological biomarkers are classified into those for susceptibility, exposure, and effect.

No single toxicological biomarker is applicable to all situations. So, several biomarkers may be used in any given situation.

Table 3 Books on biomarkers or toxicology. This table lists books relevant to biomarkers or toxicology

Book title	Authors or editors	Publisher	Year of publication
Aflatoxins in Foods: An Update	Hakeem KR, Oliveira CAF, Ismail A	Springer	2021
Basic and Clinical Toxicology of Mustard Compounds	Balali-Mood M, Abdollahi M	Springer	2015
Biomarkers in Toxicology	Gupta R	Elsevier Inc.	2014
Biomarkers in Toxicology	Gupta RC	Academic Press	2019
Chemical Warfare Toxicology	Worek F, Jenner J, Thiermann H	Royal Society of Chemistry	2016
Critical Care Nutrition Therapy for Non-Nutritionists	Berger M	Springer	2018
Critical Care Toxicology	Brent J, Burkhart K, Dargan P, Hatten B, Mégarbane B, Palmer R, White J	Springer	2020
Developmental Neurotoxicity of Lead	Winder C	Springer Netherlands	1984
Drug Discovery Toxicology: From Target Assessment to Translational Biomarkers	Will Y, McDuffie JE, Olaharski AJ, Jeffy BD	John Wiley and Sons, Inc.	2016
Drug Safety Evaluation	Gautier J-C	Springer	2017
Effects of Lead Toxicity on Growth Hormone Levels in Fish	Riaz S, Sajjad S	LAP Lambert Academic Publishing	2012
Environmental Neuroscience Advancing the Understanding of How Chemical Exposures Impact Brain Health and Disease	Bain L, Posey Norris S, Stroud C	National Academies Press	2020
Genetic Toxicology Testing: A Laboratory Manual	Proudlock R	Elsevier Science	2016
Genotoxicity Assessment, Methods and Protocols	Dhawan A, Bajpayee M	Humana Press	2013
Handbook of Systems Toxicology	Casciano DA, Sahu SC	Wiley	2011
Harmful algal blooms (HABs) in Latin America	Müller MN, Mardones JI, Dorantes-Aranda JJ	Frontiers Media SA	2020
History of Alternative Test Methods in Toxicology	Balls M, Worth A, Combes R	Elsevier Science	2018
Human Biomonitoring for Environmental Chemicals	National Research Council	National Academic Press	2006
Introduction to Mass Spectrometry, 4th Edition	Watson J, Sparkman O	John Wiley and Sons Ltd	2007
Lead and Public Health	Mushak P	Elsevier (SandT)	2011

(continued)

Table 3 (continued)

Book title	Authors or editors	Publisher	Year of publication
Lead Intoxication and Evaluation of Oxidation Stress in Human	Batool Z, Haque A, Sadiq S	LAP Lambert Academic Publishing	2013
Lead is A Silent Hazard	Stapleton R M	Walker and Co	1995
Lead Poisoning	Breen JJ, Stroup CR	Taylor and Francis Inc	2020
Lead Poisoning and Developing Organism	Jain R	LAP Lambert Academic Publishing	2015
Lead Poisoning and Lead Absorption; The Symptoms, Pathology and Prevention	Legge TM	Read Books	1912
Lead: Its Effects on Environment and Health	Sigel A, Sigel H, Sigel R	De Gruyter	2017
Mass Spectrometry - Principles and Applications, 3rd Edition	de Hoffman E, Stroobant V	John Wiley and Sons, Ltd	2007
Mass Spectrometry in Metabolomics	Raftery D	Springer	2014
Micronucleus Assay in Toxicology	Knasmuller S, Fenech M	Royal Society in Chemistry	2019
MRI: The Basics	Hashemi R, Bradley W, Lisanti C	Philadelphia: Lippicott Williams and Wilkins	2010
Pathological and Molecular Diagnosis of Lead Poisoning in Animals	Karamala S, Chinthamaneni S	LAP Lambert Academic Publishing	2017
Practical Guide for Medical Management of Chemical Warfare Casualties	Balali-Mood M, Mathews R, Pita R, Rice P, Romano J, Theirman H, Willems J	OPCW	2016
Predictive Biomarkers in Oncology: Applications in Precision Medicine	Sunil Badve S, Kumar GL	Springer	2019
Principles of Toxicology Testing	Barile F	Routledge Taylor and Francis Group	2013
Proteomic and Metabolomic Approaches to Biomarker Discovery, 2nd Edition	Issaq HJ, Veenstra TD	Academic Press	2019
Role of Oxidative Stress in Fluoride Exposure	Miranda GHN	ScienceDirect	2021
Toxicology and Combat Critical Care in Clinical Practice	Hutchings S	Springer	2016

Table 4 Techniques and platforms related to biomarkers or toxicology. This table lists technologies or platforms relevant to biomarkers or toxicology. Please note, occasionally the location of the websites or web address changes

Organization or company name	Web address
ABCAM (FirePlex)	https://www.abcam.com/kits/fireplex-high-throughput
Almac	https://www.almacgroup.com/
Applied BioPhysics	https://www.biophysics.com/
Biocept	https://biocept.com/
Biomarker Bay	https://biomarkerbay.com/
Biomarkers – ACROBiosystems	https://www.acrobiosystems.com/
Biomarkers Platform – A_IATRIS	http://www.aiatris.it/biomarkers
Brain Tumour – Brain Tumour Charity	https://www.thebraintumourcharity.org/
Celemics BTSeq™ Preparation – Whole Genome Sequencing	https://www.celemics.com/en/index.asp
CORDIS – Biomarker Discovery and Validation	https://cordis.europa.eu/programme/id/H2020_IMI2-2020-23-03
Digital Biomarker Platform – Koneksa	https://www.koneksahealth.com/platform/
Genentech	https://www.gene.com/
Human Biomonitoring for Europe (HBM4EU)	https://www.hbm4eu.eu/
Human Metabolome Database	https://hmdb.ca/
KEGG PATHWAY Database	https://www.genome.jp/kegg/pathway.html
Lead Poisoning – News Medical Life Sciences	https://www.news-medical.net/health/Lead-Poisoning-Diagnosis-and-Treatment.aspx
METLIN *Metabolite and Chemical Entity Database)	https://metlin.scripps.edu
Microfluidic platforms for biomarker analysis – PubMed	https://pubmed.ncbi.nlm.nih.gov/24663505/
NanoString Counter	https://www.nanostring.com/
Olink	https://www.olink.com/
Slovak National Platform for 3Rs	https://www.snp3rs.com/
SomaLogic	https://somalogic.com/
SynVivo	https://www.synvivobio.com/
Ultrasensitive Detection Platform of Disease Biomarkers	https://pubs.acs.org/doi/abs/10.1021/acs.analchem.0c03822

Investigations of toxicological biomarkers are ongoing and advancing at the same time.

This chapter lists resources relevant to the use of biomarkers in toxicology.

Table 5 Other resources of interest or relevance for health care professionals or patients related to biomarkers or toxicology. This table lists other resources of interest or relevance to biomarkers or toxicology. Please note, occasionally the location of the websites or web address changes

Name of resource or organization	Web address
Adult Lead – University of Rochester Medical Center	https://www.urmc.rochester.edu/encyclopedia/content.aspx?contenttypeid=167andcontentid=lead_blood_adult
BEST Resource: Harmonizing Biomarker Terminology	https://www.fda.gov/media/99221/download
Biomarkers – Taylor and Francis Online	https://www.tandfonline.com/toc/ibmk20/current
Biomarkers Consortium Resources	https://fnih.org/what-we-do/biomarkers-consortium/about/resources
Biomarkers Resources – Eurofins	https://www.eurofins.com/biopharma-services/bioanalysis/biomarker-testing-services/biomarkers-resources/
CDC's Childhood Lead Poisoning Prevention Programme	https://www.cdc.gov/nceh/lead/default.htm
EAU Patient Information	https://patients.uroweb.org/
Endocrine disrupting Chemicals 2012 – United Nations Environment Programme, WHO	https://apps.who.int/iris/handle/10665/78102
Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) Final Report – EPA	https://www.epa.gov/endocrine-disruption/endocrine-disruptor-screening-and-testing-advisory-committee-edstac-final
European Federation of Pharmaceutical Industries and Associations: Working with Patient Groups	https://www.efpia.eu/relationships-code/patient-organisations/
FDA-NIH Biomarker Working Group	https://www.fda.gov/about-fda/center-drug-evaluation-and-research-cder/fda-biomarkers-working-group
Fight Colorectal Cancer	https://fightcolorectalcancer.org/
Fluoride – Office of Dietary Supplements (ODS) of the National Institutes of Health (NIH)	https://ods.od.nih.gov/factsheets/Fluoride-HealthProfessional/
Harmful Algal Bloom (HAB)-Associated Illness – CDC	https://www.cdc.gov/habs/illness-symptoms-freshwater.html
Human Protein Atlas	https://www.proteinatlas.org/
Inadequate or Excess Fluoride – WHO	https://www.who.int/teams/environment-climate-change-and-health/chemical-safety-and-health/health-impacts/chemicals/inadequate-or-excess-fluoride
International Coordinated Research on Harmful Algae – UNESCO	https://en.unesco.org/news/international-coordinated-research-harmful-algae-and-their-impacts-society

(continued)

Table 5 (continued)

Name of resource or organization	Web address
Lead – EPA	https://www.epa.gov/lead
Lead Abatement Resource Centre (LARC)	https://larcusa.org/science/
Lead Poisoning – CDC	https://search.cdc.gov/search/index.html?query=LEAD%20POISONINGanddpage=1
Lead Poisoning – Centre for Parent Information and Resources	https://www.parentcenterhub.org/ohi-lead/
Lead Poisoning – Florida Health	http://www.floridahealth.gov/environmental-health/lead-poisoning/adults.html
Lead poisoning – Mayo Clinic	https://www.mayoclinic.org/diseases-conditions/lead-poisoning/diagnosis-treatment/drc-20354723
Lead Poisoning – News Medical Life Sciences	https://www.news-medical.net/health/Lead-Poisoning-Diagnosis-and-Treatment.aspx
Lead Poisoning Prevention – Nationwide Chlidren's	https://www.nationwidechildrens.org/family-resources-education/health-wellness-and-safety-resources/helping-hands/lead-poisoning-prevention
Lead Poisoning Treatment and Prevention – Children's Hospital at Montefiore	https://www.cham.org/programs-centers/lead-poisoning-treatment-prevention-program/resources
Lead: Science and Technology – EPA	https://www.epa.gov/lead/lead-science-and-technology
Minnesota Department of Health	https://www.health.state.mn.us/communities/environment/lead/links.html
National Biomonitoring Program, CDC	https://www.cdc.gov/biomonitoring/index.html
National HAB Committee	https://hab.whoi.edu/national-hab-committee/
National Reference Laboratory for Pesticides of the UVMP in Košice, The Slovak Republic	https://www.uvlf.sk/en/organisation-structure/self-sustaining-units/national-reference-laboratory-for-pesticides
Pharmaceutical Inspection Co-operation Scheme (PIC/s)	https://picscheme.org/en/picscheme
Pure Earth	https://www.pureearth.org/
Scanner Tests for Lead Poisoning – New Scientist	https://www.newscientist.com/article/mg12316714-800-technology-scanner-tests-for-lead-poisoning/
Series 890 – Endocrine Disruptor Screening Program Test Guidelines – EPA	https://www.epa.gov/test-guidelines-pesticides-and-toxic-substances/series-890-endocrine-disruptor-screening-program
The Patients Association	https://www.patients-association.org.uk/
Toxic Elements – Oxford Academic (Laboratory Medicine)	https://academic.oup.com/labmed/article/42/12/735/2504927

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