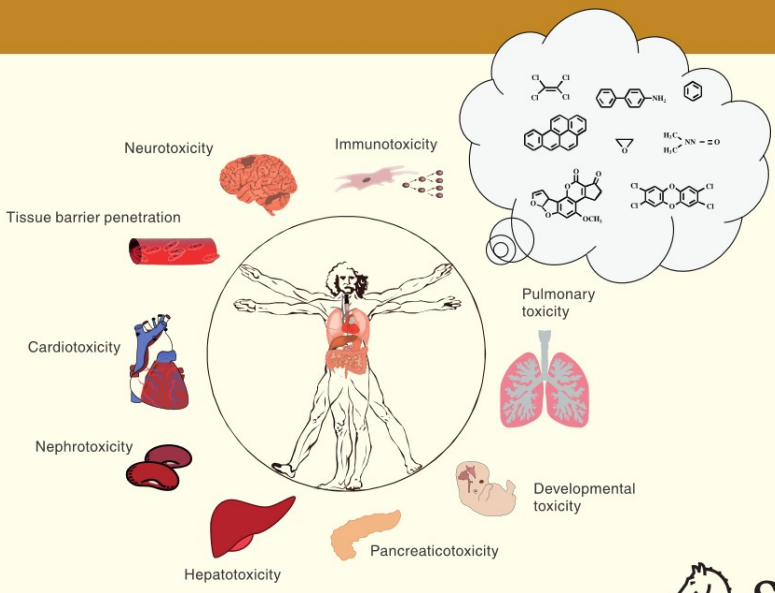
Experientia Supplementum
Volume 101

Molecular, Clinical and Environmental Toxicology

Volume 3: Environmental Toxicology

Andreas Luch
Editor



Springer

Experientia Supplementum

Volume 101

For further volumes:
http.//www.springer.com/series/4822

Andreas Luch
Editor

Molecular, Clinical and Environmental Toxicology

Volume 3: Environmental Toxicology

Springer

Editor
Andreas Luch
German Federal Institute for Risk
Assessment (BfR)
Max-Dohrn-Strasse 8-10
Berlin
Germany

ISSN 1023-294X
ISBN 978-3-7643-8339-8 ISBN 978-3-7643-8340-4 (eBook)
DOI 10.1007/978-3-7643-8340-4
Springer Heidelberg Dordrecht London New York

Library of Congress Control Number: 2012940196

Printed on acid-free paper

Springer Basel AG is part of Springer Science + Business Media (www.springer.com)

Preface

With this third and last volume the current overview on modern toxicology finally comes to an end. Certainly, as with any other living scientific discipline this is of temporary and short nature only. The progress is fast, because modern toxicological research benefits from many areas of basic sciences and medical disciplines in a unique way. Since toxicology ultimately is aimed at detecting compound-mediated harm and preventing or counteracting any damage that might result from exposures to certain compounds, applied and translational research in this field rapidly moves into clinical applications and/or public health decision making. Therefore, progress in toxicological sciences is always under special notice and usually receives broad attention and appreciation by the general public. On the other hand—of course!—tight environmental legislation triggered by public pressure not always will be welcomed from any side, in particular if analytical and technological measures required to monitor and to reduce the release of potentially harmful compounds will be of high financial investment. Still, despite some irrationality and "chemophobia" in the context of synthetic chemicals, long-term acceptance and a widespread and general public support of modern chemical industries and chemical progress will be only achievable through transparency, authoritative chemical monitoring (including biomonitoring), and reliable toxicological assessments. In addition, it will be crucial to ensure the exclusion of any considerable increases in health risks beyond the background levels that have been established and people were gotten adapted to during the absence of the chemicals and products that are intended to be newly introduced to the consumer market.

It came without much surprise that the entire book project finalized today turned out as a great challenge and to cover almost all of the relevant fields and aspects of this great interdisciplinary scientific discipline was not always easy. Everything relied on the efforts and dedication of individuals who were willing to contribute with excellent and comprehensive reviews on particular topics in modern toxicology. Altogether it was possible to gather more than 100 experts covering all fields and expertise required for this comprehensive three-volume overview on *Molecular, Clinical & Environmental Toxicology*.

The third and last volume focuses on environmental toxicology and encompasses topics and certain kinds of chemical classes that have gained importance in toxicological research due to their inherent biological activity and their abundance in the human and natural environment. At the same time, more recent and "modern" research areas in the fields of, for instance, toxicity of mixtures, nanotoxicology, endocrine-disrupting chemicals, low-dose effects, and epigenetics will also be discussed in the present volume. My deep gratitude goes to all of the authors who were highly engaged and focused to deliver a nice piece of work and to make this project finally successful.

I believe that advanced students and instructors of toxicology and public health will benefit from this overview on modern toxicology and toxicological research, which, in great parts, emphasizes and illuminates molecular mechanisms and toxicokinetics rather than just providing descriptive accounts. With this, it contributes to the distribution of established scientific knowledge in the community and therefore will help to lay the ground for a more serious and science-based discussion of the adverse health effects of synthetic chemicals and biogenic products, thereby leading to a greater rationality in the assessment of health and environmental risks that may be associated with the exposure against such compounds.

At the end, I again want to express my special gratitude toward the publishing house, and here in particular to Dr. Beatrice Menz, who was very focused and propulsive in pushing the editor over the finishing line. Honestly, my special thanks for this to her in a personally difficult and wasting period of time in my life.

Berlin, Germany Andreas Luch

Contents

Chemical Hazards in the Organisation 1
Chris Winder

Toxicology of Water 21
Xiaowei Zhang, Steve Wiseman, and John Giesy

Perfluorinated Compounds 47
Christopher Lau

Toxicologically Relevant Phthalates in Food 87
Oliver Kappenstein, Bärbel Vieth, Andreas Luch, and Karla Pfaff

Exposure to Polycyclic Aromatic Hydrocarbons: Bulky DNA Adducts and Cellular Responses 107
Frank Henkler, Kristin Stolpmann, and Andreas Luch

Heavy Metal Toxicity and the Environment 133
Paul B. Tchounwou, Clement G. Yedjou, Anita K. Patlolla, and Dwayne J. Sutton

Toxicology of Ambient Particulate Matter 165
Damiën van Berlo, Maja Hullmann, and Roel P.F. Schins

Nanomaterials: A Challenge for Toxicological Risk Assessment? 219
Andrea Haase, Jutta Tentschert, and Andreas Luch

Immunotoxicology and Its Application in Risk Assessment 251
Andrew A. Rooney, Robert W. Luebke, MaryJane K. Selgrade, and Dori R. Germolec

Chemical Sensitization and Allergotoxicology 289
Rosette L. Van Den Heuvel, Nathalie Lambrechts, Sandra Verstraelen, Inge C. Nelissen, and Greet E.R. Schoeters

Male Reprotoxicity and Endocrine Disruption 315
Sarah Campion, Natasha Catlin, Nicholas Heger, Elizabeth V. McDonnell, Sara E. Pacheco, Camelia Saffarini, Moses A. Sandrof, and Kim Boekelheide

Interspecies Uncertainty in Molecular Responses and Toxicity of Mixtures 361
Moiz M. Mumtaz and Hana R. Pohl

Recent Trends in Statistical QSAR Modeling of Environmental Chemical Toxicity 381
Alexander Tropsha

Chirality and Its Role in Environmental Toxicology 413
Imran Ali, Hassan Y. Aboul-Enein, Mohd Marsin Sanagi, and Wan Aini Wan Ibrahim

Genetic Variability in Molecular Responses to Chemical Exposure 437
Chirag J. Patel and Mark R. Cullen

Biomarkers in Toxicology and Risk Assessment 459
Bruce A. Fowler

A Personalized Life: Biomarker Monitoring from Cradle to Grave 471
Harald Jungnickel and Andreas Luch

On the Role of Low-Dose Effects and Epigenetics in Toxicology 499
Lena Smirnova, Alexandra Sittka, and Andreas Luch

Hormesis: Improving Predictions in the Low-Dose Zone 551
E.J. Calabrese

Index 565

List of Contributors

Hassan Y. Aboul-Enein Pharmaceutical and Medicinal Chemistry Department, Pharmaceutical and Drug Industries Research Division, National Research Centre, Dokki, Cairo 12311, Egypt, haboulenein@yahoo.com

Imran Ali Department of Chemistry, Jamia Millia Islamia (Central University), New Delhi, India

Damiën van Berlo Particle Research, Institut für Umweltmedizinische Forschung (IUF), Heinrich-Heine University Düsseldorf, Auf'm Hennekamp 50, 40225 Düsseldorf, Germany

Kim Boekelheide Department of Pathology and Laboratory Medicine, Brown University, Providence, RI 02912, USA, kim_boekelheide@brown.edu

Edward J. Calabrese Department of Public Health, University of Massachusetts, Amherst, MA, USA; Department of Toxicology, Environmental Health Sciences, University of Massachusetts, Morrill I, N344, Amherst, MA 01003, USA, edwardc@schoolph.umass.edu

Sarah Campion Department of Pathology and Laboratory Medicine, Brown University, Providence, RI 02912, USA

Natasha Catlin Department of Pathology and Laboratory Medicine, Brown University, Providence, RI 02912, USA

Mark R. Cullen Department of Medicine, Stanford University, 1265 Welch Road, X388, Stanford, CA 94305, USA, mrcullen@stanford.edu

Bruce A. Fowler Division of Toxicology and Environmental Medicine, Agency for Toxic Substances and Disease Registry (ATSDR), Atlanta, GA 30333, USA; ICF International, Fairfax, VA, USA, bfowler@icfi.com; drtox@earthlink.net

Dori R. Germolec National Toxicology Program, Toxicology Branch, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA

John Giesy Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan, Canada; Department of Veterinary Biomedical Sciences, University of Saskatchewan, Saskatoon, Saskatchewan, Canada; Department of Biology and Chemistry, City University of Hong Kong, Kowloon, Hong Kong, China, John.Giesy@usak.ca

Andrea Haase Department of Product Safety, German Federal Institute for Risk Assessment (BfR), Max-Dohrn-Strasse 8-10, 10589 Berlin, Germany

Nicholas Heger Department of Pathology and Laboratory Medicine, Brown University, Providence, RI 02912, USA

Frank Henkler Department of Product Safety, German Federal Institute for Risk Assessment (BfR), Max-Dohrn-Strasse 8-10, 10589 Berlin, Germany

Rosette L. Van Den Heuvel Environmental Risk and Health Unit—Toxicology, Flemish Institute for Technological Research (VITO N.V.), Centre for Advanced R&D on Alternative Methods (CARDAM), Boeretang 200, 2400 Mol, Belgium, rosette.vandenheuvel@vito.be

Maja Hullmann Particle Research, Institut für Umweltmedizinische Forschung (IUF), Heinrich-Heine University Düsseldorf, Auf'm Hennekamp 50, 40225 Düsseldorf, Germany

Wan Aini Wan Ibrahim Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 81310, UTM Johor Bahru, Johor, Malaysia

Harald Jungnickel Department of Product Safety, German Federal Institute for Risk Assessment (BfR), Max-Dohrn-Strasse 8-10, 10589 Berlin, Germany, Harald.Jungnickel@bfr.bund.de

Oliver Kappenstein Department of Product Safety, German Federal Institute for Risk Assessment (BfR), Max-Dohrn-Strasse 8-10, 10589 Berlin, Germany, Oliver.Kappenstein@bfr.bund.de

Nathalie Lambrechts Environmental Risk and Health Unit—Toxicology, Flemish Institute for Technological Research (VITO N.V.), Centre for Advanced R&D on Alternative Methods (CARDAM), Boeretang 200, 2400 Mol, Belgium

Christopher Lau Toxicity Assessment Division, National Health and Environment Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711, USA, lau.christopher@epa.gov

Andreas Luch Department of Product Safety, German Federal Institute for Risk Assessment (BfR), Max-Dohrn-Strasse 8-10, 10589 Berlin, Germany, Andreas.Luch@bfr.bund.de

Robert W. Luebke National Health and Environmental Effects Research Laboratory, Cardiopulmonary and Immunotoxicology Branch, U.S. Environmental Protection Agency, Research Triangle Park, NC, USA

Elizabeth V. McDonnell Department of Pathology and Laboratory Medicine, Brown University, Providence, RI 02912, USA

Moiz M. Mumtaz Division of Toxicology and Human Health Sciences, Agency of Toxic Substances & Disease Registry (ATSDR), Centers for Disease Control and Prevention (CDC), U.S. Department of Health and Human Services, Atlanta, GA 30333, USA, mgm4@cdc.gov

Inge C. Nelissen Environmental Risk and Health Unit—Toxicology, Flemish Institute for Technological Research (VITO N.V.), Centre for Advanced R&D on Alternative Methods (CARDAM), Boeretang 200, 2400 Mol, Belgium

Sara E. Pacheco Department of Pathology and Laboratory Medicine, Brown University, Providence, RI 02912, USA

Chirag J. Patel Department of Medicine, Stanford University, 1265 Welch Road, X338, Stanford, CA 94305, USA, cjp@stanford.edu

Anita K. Patlolla NIH-RCMI Center for Environmental Health, College of Science, Engineering and Technology, Jackson State University, 1400 Lynch Street, Box 18750, Jackson, MS 39217, USA

Karla Pfaff Department of Product Safety, German Federal Institute for Risk Assessment (BfR), Max-Dohrn-Strasse 8-10, 10589 Berlin, Germany

Hana R. Pohl Division of Toxicology and Human Health Sciences, Agency of Toxic Substances & Disese Registry (ATSDR), Centers for Disease Control and Prevention (CDC), U.S. Department of Health and Human Services, Atlanta, GA 30333, USA, hpohl@cdc.org

Andrew A. Rooney National Toxicology Program, Office of Health Assessment and Translation, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA, Andrew.Rooney@nih.gov

Camelia Saffarini Department of Pathology and Laboratory Medicine, Brown University, Providence, RI 02912, USA

Mohd Marsin Sanagi Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 81310, UTM Johor Bahru, Johor, Malaysia

Moses A. Sandrof Department of Pathology and Laboratory Medicine, Brown University, Providence, RI 02912, USA

Roel P.F. Schins Particle Research, Institut für Umweltmedizinische Forschung (IUF), Heinrich-Heine University Düsseldorf, Auf'm Hennekamp 50, 40225 Düsseldorf, Germany, roel.schins@uni-duesseldorf.de

MaryJane K. Selgrade Environmental Risk and Toxicology, ICF International, Durham, NC 27713, USA

Alexandra Sittka Department of Product Safety, German Federal Institute for Risk Assessment, Max-Dohrn-Strasse 8-10, 10589 Berlin, Germany

Lena Smirnova Department of Product Safety, German Federal Institute for Risk Assessment, Max-Dohrn-Strasse 8-10, 10589 Berlin, Germany

Greet E.R. Schoeters Environmental Risk and Health Unit—Toxicology, Flemish Institute for Technological Research (VITO N.V.), Centre for Advanced R&D on Alternative Methods (CARDAM), Boeretang 200, 2400 Mol, Belgium

Kristin Stolpmann Department of Product Safety, German Federal Institute for Risk Assessment (BfR), Max-Dohrn-Strasse 8-10, 10589 Berlin, Germany

Dwayne J. Sutton NIH-RCMI Center for Environmental Health, College of Science, Engineering and Technology, Jackson State University, 1400 Lynch Street, Box 18750, Jackson, MS 39217, USA

Paul B. Tchounwou NIH-RCMI Center for Environmental Health, College of Science, Engineering and Technology, Jackson State University, 1400 Lynch Street, Box 18750, Jackson, MS 39217, USA, paul.b.tchounwou@jsums.edu

Jutta Tentschert Department of Product Safety, German Federal Institute for Risk Assessment (BfR), Max-Dohrn-Strasse 8-10, 10589 Berlin, Germany

Alexander Tropsha Laboratory of Molecular Modeling, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA, alex_tropsha@unc.edu

Sandra Verstraelen Environmental Risk and Health Unit—Toxicology, Flemish Institute for Technological Research (VITO N.V.), Centre for Advanced R&D on Alternative Methods (CARDAM), Boeretang 200, 2400 Mol, Belgium

Bärbel Vieth Department of Product Safety, German Federal Institute for Risk Assessment (BfR), Max-Dohrn-Strasse 8-10, 10589 Berlin, Germany

Chris Winder Faculty of Business, Australian Catholic University, Level 10 Tenison Woods House, 8-20 Napier Street, North Sydney, NSW 2060, Australia, Chris.Winder@acu.edu.au

Steve Wiseman Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, steve.wiseman@usask.ca

Clement G. Yedjou NIH-RCMI Center for Environmental Health, College of Science, Engineering and Technology, Jackson State University, 1400 Lynch Street, Box 18750, Jackson, MS 39217, USA

Xiaowei Zhang State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing, China, howard 50003250 @yahoo.com

Chemical Hazards in the Organisation

Chris Winder

Abstract The use of hazardous chemicals in organisations represents a substantial risk to occupational health, safety and the environment (OHSE). Organisational directors and managers have a responsibility to provide and maintain organisational management systems that manage these risks. The risk management approach of establishing organisational considerations, identifying chemical hazards (health and environmental), assessing and controlling risks and evaluating management activities has become the *de facto* means of managing organisational hazards in general and may be satisfactorily applied to the management of chemicals in the organisation. The Globally Harmonized System for the Classification and Labelling of Chemicals (GHS) is now at the forefront of major regulatory issues facing the chemicals manufacturing industry and downstream users of chemicals. The GHS offers one system for the classification of all dangerous, toxic and environmental (ecotoxic) effects of chemicals. Organisations should develop occupational health, safety and environment (OHSE) management systems which contain programs and procedures that contain systems for inventory control, hazard communication, competency training, risk assessment and control, transport and storage, monitoring and health surveillance, chemical emergencies (including accident investigation), waste minimisation and disposal, record keeping and management system review.

Keywords Globally harmonised system · Hazardous chemicals · Occupational toxicology · Occupational health and safety management · Occupational health, safety and environment

C. Winder (✉)
Faculty of Business, Australian Catholic University, Level 10 Tenison Woods House, 8-20 Napier Street, North Sydney, NSW 2060, Australia
e-mail: Chris.Winder@acu.edu.au

A. Luch (ed.), *Molecular, Clinical and Environmental Toxicology*,
Experientia Supplementum 101, DOI 10.1007/978-3-7643-8340-4_1,

Introduction

Chemicals are found in workplaces of many organisations. A significant proportion of them are harmful to health and/or the environment and need to be identified, assessed, controlled or otherwise managed to ensure a safe and healthy work environment exists for all who work or visit those organisations, and that environmental impacts are minimised.

Operationally, the term chemical is usually used to cover those materials that are natural or artificial entities, composite materials, mixtures or formulations, other than articles. Of course, from an occupational, health, safety and environment (OHSE) perspective, all chemicals are not necessarily the problem: only those with hazards or risks.

There are two factors that affect chemical safety:

- Inherent properties (toxicity, flammability, corrosiveness, destruction of the environment and so forth)
- Factors relating to exposure (e.g. the probability of exposure, duration, frequency and intensity of exposure during or after use, or following dispersal in the environment)

The Globally Harmonized System for the Classification and Labelling of Chemicals

A major problem with the sound management of chemicals is the different descriptions that chemicals with hazards are given and the variety of definitions provided in formal, regulatory documents, for example dangerous goods [1], hazardous substances [2], poisons [3] and environmentally toxic chemicals or hazardous wastes [4]. All such chemicals may be classified using different endpoints and varying criteria.

The varying classification systems across different regulatory jurisdictions create confusion for many users of chemicals. Problems between the two largest classification schemes used worldwide, the UN Dangerous Goods System and the classification system in use in the European Community, created problems such as international friction and non-tariff barriers to trade.

From this, a Globally Harmonized System for the Classification and Labelling of Chemicals (GHS) was completed in 2002 [5]. Under the GHS, there will be one system for the classification of all dangerous, toxic and environmental (ecotoxic) effects of chemicals—a system that should go some way to abolishing the confusion that the different categories of chemicals creates. Importantly, categories of chemicals with various hazard properties (such as dangerous goods, hazardous substances, poisons, environmentally hazardous chemicals and so forth) will be replaced by one category: hazardous chemicals.

The main GHS elements are [5, 6]:

- Classification criteria for substances and mixtures for:
  - States of matter
  - Physical effects
  - Toxic (health) effects
  - Environmental effects
- Requirements for hazard communication for chemicals, such as:
  - Labels
  - Safety data sheets (SDS)

GHS Classification Criteria

States of Matter

Before classifying the various physical, toxic and environmental endpoints, the GHS classifies the states of matter:

- Gas—a chemical that is completely gaseous or has a vapour pressure at 50°C above 3 kPa
- Liquid—not a gas but a chemical with an initial melting point below 20°C
- Solid—all other substances

Physical Effects

Physical hazards are based on the classification criteria of the UN Dangerous Goods System and include criteria for:

- Explosiveness
- Gases under pressure
- Liquefied gases
- Dissolved gases
- Water-activated flammable gases
- Flammable gases
- Flammable liquids
- Flammable solids
- Self-reactive substances
- Pyrophoric liquids and solids
- Self-heating substances
- Dangerous when wet substances
- Oxidising liquids and solids
- Oxidising peroxides
- Corrosive substances or mixtures to materials

Note that radioactivity (present in the dangerous goods classification) is absent from this classification.

Toxic Effects

Toxicity (health) hazards are based on those on the classification criteria of the European Union:

- Single dose toxicity
- Skin irritation and corrosion
- Eye irritation and serious eye damage
- Skin sensitisation or respiratory sensitisation
- Repeated dose toxicity
- Target organ systemic toxicity (TOST)
- Genotoxicity and germ cell toxicity
- Reproductive effects, developmental effects
- Carcinogenicity

Note that infectious hazards (present in the dangerous goods classification) are absent from this classification.

Environmental Effects

Environmental hazards in the GHS only really address aquatic toxicity and include:

- Single dose toxicity
- Repeated dose toxicity
- Ready degradability
- Bioaccumulation and bioconcentration factor (BCF)

Systems for terrestrial or ecosystem toxicity are yet to be developed for the GHS.

GHS and Hazard Communication

Hazard communication for chemicals has always been a vexed problem, as different forms of information are required for different types of individuals, for example users, workers, consumers, emergency responders and chemical handlers (transport, storage personnel and so on) have different information needs. However, once a chemical has been identified as hazardous and classified as to the type of hazard (s), providing hazard communication is the next step.

The GHS will facilitate hazard communication through:

- Container labels containing:
  - Hazard symbols/pictograms
  - Signal words (Danger, Warning)
  - Hazard statements (standardised and linked with signal words and hazard symbols)
  - Precautionary statements (a standardised set of statements is being developed)

- Safety data sheets (SDS) to a standard 16 header format:
  - Identification of the material supplier
  - Hazard(s) identification
  - Composition/information on ingredients
  - First aid measures
  - Fire-fighting measures
  - Accidental release measures
  - Handling and storage
  - Exposure controls and personal protection
  - Physical and chemical properties
  - Stability and reactivity
  - Toxicological information
  - Ecological information
  - Disposal considerations
  - Transport information
  - Regulatory information
  - Additional information not otherwise covered

Organisations, Hazardous Chemicals and Risk Management

Duty of Care

It is a commonly held belief among business people that the function of business is to make profits and create wealth for its shareholders. Such a viewpoint does not acknowledge the principle that businesses draw a licence to operate from the community, and that businesses have an obligation to that community. Increasingly, the 'licence to operate' requires that businesses acknowledge they have a duty of care to the community and to the environment, and to operate to high standards of corporate responsibility. Duty of care must be demonstrated by evidence of systems for due diligence: Failure to make this effort may be negligence.

Hazardous Chemicals Management

This has placed pressure on employers and workplace managers to better manage hazardous chemicals within their organisations [7]. These activities, which impact on all aspects of the life cycle of a chemical, are found in various parts of an organisation, from purchase, transport, storage, use, wastes disposal and the like [8].

However, a lack of expertise, resources and willingness in employers (especially those in small businesses) often creates difficulties in identifying and tackling health, safety and environmental issues. External pressures such as the economy, competitiveness or political priorities can further complicate the management of OHSE.

Hazardous Chemicals and Risk Management

Where some of the activities carried out by organisations have an adverse impact (whether OHS, environment, security or any one of a substantial number of risks), it is the role of the organisation to identify the various adverse consequences, assess their impact(s) and take steps to minimise such impacts. More recently, such activities fall under the general scope of risk management [9]. This offers a means of dealing with these problems, as it provides a more proactive and considered approach to develop a framework of measures to consult with employees; identify, assess and control organisational chemical hazards; and review the effectiveness of the measures.

Systems are required to ensure that OHSE hazards are identified, risk assessment procedures are in place, the right controls are used and emergency procedures are in place. In turn, this requires good management [10]. The risk management approach of establishing organisational considerations, identifying chemical hazards (health and environmental), assessing risks, controlling risks and evaluating management activities has become the de facto means of managing organisational hazards in general and may be satisfactorily applied to the management of chemicals at in organisations [10–12].

Risk management involves a range of activities. It is important to recognise that most, if not all, chemicals can be handled and used safely within organisations, providing:

- The hazards are known and understood
- Correct handling and use procedures are in place and adhered to
- The correct equipment to handle and use chemicals is available, used and maintained
- Employees are informed about hazards and trained in correct procedures
- Prompt action is taken to control and minimise problems that do arise

In general, risk management requires the establishment of risk management systems that outline the obligations of managers, occupiers, employees and suppliers of materials. Boundaries need to be established, that is, will the risk management approach apply to (among other things):

- Selected OHS (such as dusts) or environmental (e.g. wastes) issues, or
- Only OHS hazards or only environmental impact, or
- A fully integrated program that identifies all OHS and environmental hazards and impacts

More specific activities are then undertaken in accordance with the following framework.

Identification of Hazardous Chemicals

- Criteria for determining a hazardous chemical [2, 6]
- Requirements for hazard communication on chemical hazards, such as labels, SDS and registers [6]
- Requirements for competency training of employees

Assessment of the Risks of Hazardous Chemicals

- Procedures for qualitative organisational assessment where chemicals are used covering both OHS and environmental aspects
- Procedures for quantitative workplace assessment where qualitative assessments identify unknown, potentially unacceptable or inadequately controlled risks

Control of Hazardous Chemicals That Are a Risk to Health or Environment

- Requirements for control of exposure or impact where risk assessments indicate that problems to exposed workers, the public or the environment exist
- Consideration of the need for organisational or environmental monitoring
- Consideration of the need for health surveillance of employees
- Systems for emergency response, including explosions, fires, spills or first aid incidents
- Systems for environmental management

Review of Management Systems and Operational Activities

- Incident reporting
- Internal and external audit procedures
- Requirements for record keeping
- Procedures for management review

In short, risk management provides an approach by which chemicals in the organisation can be managed safely [10].

Developing a Hazardous Chemicals Management System

Most senior managers cannot reasonably expect to be informed about every issue and decision within the organisation. In view of this, meeting OHSE chemical safety responsibilities towards their employees and the environment can be problematic. Some organisations nominate a person who is responsible for organisational safety and/or environmental impacts, and presumably, they are then able to deal with chemical safety problems.

The management of OHSE can be facilitated by a chemical safety management system comprising the following areas [7, 10], which are outlined in more detail in the following sections.

- The chemical safety policy
- The chemical safety management system. This may be stand-alone or integrated into other management structures (such as an OHS, environmental, business continuity or organisational risk management system)
- Programs and subprograms dealing with specific organisational chemicals issues (such as hazard communication, risk assessment, storage, disposal)
- Procedures for those operations for which the information in the policy and program is not detailed or definitive enough
- Strategic planning and integration

Chemical safety must be included in the strategic planning process to bring it into line with all other activities of the organisation and must be compatible with other systems in the organisation.

The chemical safety management system has a number of attributes and should:

- Be focused on priorities, with selection of strategies and activities of the organisation dependent on management imperatives, organisational priorities and resource availability. Policies and programs need to be identified, developed and implemented
- Optimise the available resources on the operational side of managing the chemical safety system. Hazards and risks need to be identified through such things as organisational risk assessments, OHSE compliance activities, accident/incident investigation, review of rehabilitation and workers compensation cases, and emergencies
- Be sensitive to changes, both in the internal and external environments. Changes in the external environment offer threats to the unprepared manager and opportunities to the visionary manager. Being aware of such changes will assist in positioning the management system to gain opportunities or avoid threats

Responding to changes in the internal environment will also assist in the effective management of the system. Management audits, regular assessment of the organisation and accident statistics will assist this process.

The Chemical Safety Policy

An OHSE policy, developed in conjunction with the employees and other relevant stakeholders, defines the organisation's goals and communicates senior management's commitment. It should provide guidelines on how OHSE is to be managed within the organisation. A chemical safety policy can support this principle document. While it is possible to have stand-alone OHS or environmental policies, it is usually better to have a combined policy.

The content of a chemical safety policy would typically include the purpose, scope, measurable targets and objectives, and actions and responsibilities.

The Chemical Safety Management System

The management system should encompass all the relevant risks to health and impacts to environment that may arise from organisational activities involving chemicals (note: the term here is *chemicals*, not hazardous chemicals). The system will bring together all existing chemically related programs (including environmental impacts) and develop other programs or subprograms that are identified as missing but necessary. Note that it is not necessary to develop all new programs or subprograms. Some, such as risk assessments or emergency procedures, can be included in already existing generic risk assessment and control or emergency preparedness programs.

Chemical Safety Programs

The framework of the management system is one thing. What it deals with is another. Any matter that falls into the scope of a management system will need further systems for development, delivery and review. Of necessity, these will need to outline the ways the policy will be delivered, including the steps and organisational requirements, which assist in that process.

Programs (or in some cases, subprograms) fulfil these roles. Examples of such programs are:

- A consultation subprogram would set out roles and responsibilities of representatives, meeting schedules and outcomes, or
- A planned maintenance program would outline the broad processes required for ensuring that plant and equipment remains operational, or compliance activities such as reporting of environmental releases

The chemical safety programs, which usually form the basic elements of a chemical safety management system, relate to:

- Toxic or dangerous chemicals, especially those materials specific to the company
- Normal operational activities involving chemicals
- Near hits, incidents and accidents involving chemicals
- Emergencies involving chemicals
- Rehabilitation of injured employees
- Workers compensation
- Environmental aspects and impacts

It is important to ensure that the program is focused on the priority hazards within an organisation and that it is sensitive to changes that may occur.

The content of any individual program may include the following:

- Referral to the policy and overall commitment to the management system
- Responsibilities and authorisations
- Relevant skills/training
- Relevant equipment
- Operational requirements (including protection of employees or the environment)
- Recommended sequence of activities
- Other requirements (e.g. clean-up or record keeping)
- Referral to other programs or dates of review where relevant

Where developed and implemented, each program, within the chemical safety management system that has been identified as necessary in a particular organisation, may need procedures, specifications and work instructions (many, but not necessarily all, in written form) on how the programs are to be delivered and to ensure compliance with their requirements. These are the procedures.

Procedures

As noted above, the chemical safety policy is a statement of intent and is generally worded in broad terms. The management system and the programs it contains specify individual activities to which the policy will apply (e.g. the training program elements of the overall chemical safety program).

Some activities, however, require more detail. Job specifications, safe working procedures and the like require a detailed explanation and, in some cases, quite complicated instructions. For such tasks, procedures (normally written or delivered in specific training sessions) are required.

In some cases, a procedure can be performance based (e.g. a lone employee policy might specify that an employee has to inform someone that he or she is working alone and nominate a contact to ensure that someone will notice if the employee is absent for a longer period than anticipated). However, most procedures need to be fairly specific (e.g. a batch card for the formulation of paint in a paint factory that sets out the materials, processes and steps to be followed).

When developing or modifying procedures for inclusion in the chemical safety management system (this can be an extraordinarily complex task as many organisations have thousands of procedures), a determination needs to be made about the safety content in such documents or training materials.

Where a reference to safety or environmental protection is absent, it can be added. If there are many different procedures for the same basic process, these can be harmonised. Where safety directions for a job may be found in another document, these can be integrated into the procedures or training materials.

The Strategic Plan

The fifth principal concept of a chemical safety management system is embedding of the system into processes for strategic planning and integration. Strategic plans relate directly to corporate objectives and indicate the forms of activity needed to achieve successful outcomes for the organisation. Strategies integrate the chemical safety management program into all the management systems of the organisation and are directly identifiable with the achievement of one or more corporate objectives.

Policy, programs and procedures can be prepared for each aspect or problem identified in the risk assessment process, on a needs basis. The strategies should aim to meet the chemical safety management system objectives and be:

- Consistent with legislative compliance as a minimum
- Consistent with the chemical safety policy
- Fully documented
- Aimed at managing significant organisational chemical risks to safety or the environment

They should also include options for control/prevention of exposure of people or the environment to hazardous chemicals and other requirements of the organisation (e.g. those related to the business, customers, shareholders and the public).

Components of a Hazardous Chemicals Management System

At the operational level, chemicals control activities located in a chemicals management system are achieved through programs for (at least):

- Inventory control
- Hazard communication
- Competency training
- Risk assessment
- Risk control
- Transport and storage
- Monitoring and health surveillance

- Chemical emergencies (including accident investigation)
- Waste minimisation and disposal
- Record keeping
- Management system review

Inventory Control

Proper identification of chemical hazards in the organisation begins with establishing the numbers and types of chemicals present through creation of a chemicals inventory. Other information, such as storage locations, amounts stored and so forth, can also be collected.

Once established, the inventory can be used to identify chemicals that are out of date or surplus to requirements. These can then be discarded.

The inventory should also be used to make decision about purchasing chemicals. For example, it can be used to select less hazardous products.

Hazard Communication

All people who use or are exposed to chemicals need to be aware of the hazards of such chemicals and the recommended precautions to eliminate or control chemical risks, through provision of relevant information. In the workplace, hazard communication is usually in the form of labels on chemical containers or safety data sheets (SDS, formerly called Material Safety Data Sheets). These, and other forms of hazard communication include:

- Labels
- Safety data sheets
- Chemicals registers
- Risk assessments
- Safe working procedures

Competency Training

All persons involved in the use or handling of chemicals should be familiar with the requirements of those standards appropriate to the chemicals and processes used and with the requirements of the relevant regulatory authorities. This requirement is not just about giving employees information but providing skills so that they may be competent in the work they carry out. Competency training in safety and environmental protection is now an important part of OHSE.

For chemicals, providing information to and training workers about chemical hazards is an integral part of a program for organisation chemical safety. But this

should be reinforced with training. This should provide employees with the knowledge, skills and competencies needed to apply the information provided to them and to use the control measures, personal protective equipment and emergency procedures provided for their protection. This training may be specific to chemicals, or part of other safety training.

Training may be delivered in a number of ways and may include the following:

- Induction training
- Toolbox briefings
- On-the-job training
- Classroom training
- Online training
- Refresher training

Training and education should also enable participation in decisions about the use and handling of chemicals at work.

Risk Assessment

Assessment of the hazards and risks associated with the use of chemicals and the nature and cause of chemical-related injury, disease and environmental damage is an important factor in prevention of chemically related safety, health and environmental problems. While some chemicals risk assessment tasks seem too broad, assessing the potential effects of chemicals on workers and the environment is a specific duty imposed on organisational directors and managers by legislation.

Appropriate controls, procedures and precautions must be developed, implemented and enforced if the risks from chemicals are to be adequately contained.

But risk assessments seem like a lot of work, especially where an organisation has a lot of chemicals. And in some cases, they may not seem necessary, so a nested series of risk assessment types should be considered:

- Where the chemical has a low hazard, and there is little exposure, it is ridiculous to carry out a formal risk assessment, and a simple *notation* in the inventory or chemicals register may suffice for the purposes of risk assessment.
- Where a chemical has some hazard, but it is well understood and its risks are easily controlled by the existing organisational systems for chemicals, a *simple and obvious assessment* may be all that is needed.
- Where a group of chemicals have the same type of hazard and are used in the same way across an organisation, a *generic assessment* may be required.
- Where a number of chemicals are used in a task or process, a *task-based* or *process-based assessment* may be carried out.

All these are types of risk assessments. This allows assessment of risks for all chemicals to be dealt with appropriately and allows appropriate allocation of resources to the problem chemicals that need a formal risk assessment.

Therefore, the risk assessment process is basically quite straightforward. A stepwise approach for a suitable and sufficient assessment is recommended and can be followed quite simply [13]:

- Identify hazardous chemicals in use by the organisation
- Obtain information
- Review information
- Identify exposures (including employees, the public and the environment)
- Review current exposure controls
- Assess risks
- Control unacceptable risks
- Monitor the organisation and/or workers and/or the environment
- Keep records
- Review the risk assessment system for effectiveness

In the first instance, qualitative assessments should be used. This type of assessment will be applicable to most organisational chemical risks.

Where an assessment identifies that a risk is unacceptable, it must be eliminated or controlled, and if control is required, this should be conducted using the hierarchy of controls. For those risks that cannot be assessed using qualitative methods, the quantitative methods of occupational hygiene are available.

Risk Control

If the risk of exposure to chemicals is assessed as being unacceptable, then such risks must be eliminated or where it cannot be eliminated they must be controlled using means that are commensurate with the risk. Risk control options should be identified using the hierarchy of controls. Common options for safe use of chemicals are outlined below.

Substitution

Substituting a hazardous chemical for a less hazardous chemical is a useful way in reducing risks.

Engineering Controls

Engineering controls include total or partial enclosure, ventilation and so on. Where engineering controls are used to reduce exposure, they should be designed, installed, used and maintained properly.

Operating Instructions and Procedures

Where written instructions and procedures are used, they should be available to indicate the following:

- The need for chemicals to be checked and identified on arrival
- Recommended storage arrangements, for example, segregation where necessary
- The availability, prior to commencement of operations or delivery of the chemical, of product safety data sheets on the chemicals to be handled
- Any restrictions to be observed for particular chemicals
- Safe and correct operating instructions for the equipment in use
- Hygiene and safety procedures for all regular operations, including plant maintenance
- Procedures for any foreseeable emergencies which may arise, including the handling of spillage and any medical supervision required

Personal Protective Equipment

While personal protective equipment (PPE) is not a preferable chemical control option and is low down on the hierarchy of controls, it is an important control where exposures cannot be controlled using other controls. Selection of PPE should be to relevant standards.

Once selected, workers using PPE should be trained in proper use. PPE should be used and maintained properly for effective control. Where appropriate, combinations of the options above can be used to increase the effectiveness of control of exposure.

Transport and Storage

Chemicals, and particularly hazardous chemicals, require transport and storage. In some cases, transport and storage requirements are specified in, for example, Dangerous Goods or Poisons legislation. These are supported by a range of codes and standards for specific chemicals or groups of chemicals.

Organisations should consider not only the movement of materials into and out of their premises but also movement of chemicals within premises.

With regard to storage, a well-planned layout of storage areas is essential and should be arranged so that chemicals can be moved from one location or operation to the next with a minimum of handling.

The stacking and storage of chemicals requires constant attention so that no facet of the operations presents hazards or risks to employees or to other persons in the area. Both temporary and permanent storage should be neat and orderly. Materials

piled haphazardly or strewn about increase the possibility of personal injury, property damage or environmental impact.

Storage areas must take account of requirements for delivery, administration, production and transport, and the nature of the materials stored adjacently.

All parts of buildings and structures should be capable of safely withstanding all loads and forces imposed by the storage of materials.

Monitoring and Health Surveillance

Monitoring of systems is not preventive. However, it will advise on the suitability and success of existing control procedures. Chemicals-related disease and injury and environmental damage are prevented by minimising employee or environment exposure. Workplace monitoring can be used to check the effectiveness of exposure control measures. This can be done in a number of ways but is essentially measurement of contaminants in either the organisation or at the employee level. Monitoring may be of three types:

- Monitoring of the organisation or environment through environmental monitoring
- Monitoring of employees through health surveillance (this encompasses both biological monitoring and medical surveillance)
- Monitoring of the impact of the organisation on the environment

Chemical Emergencies

Another specific program is that related to emergency procedures. Chemical emergencies include personal injuries, fires, explosions and spills/leaks. These can be included in an organisation's emergency planning arrangements but may need to contain specific chemically related procedures, for example for appropriate treatments of chemical exposures, antidotes to poisons, spill kits or special extinguishers. Prevention and management of chemical incidents requires:

- Pre-planning
- Information systems, such as placards and emergency plans
- Specific procedures for high-risk chemicals, for both health and environmental consequences
- Training of the emergency response team
- Consultation with the emergency services

Organisational emergencies involving chemical substances can result in injury to employees and emergency response personnel, interruption of business, bankruptcy, loss of markets, environmental damage, and costly decontamination and clean-up.

Such emergencies have the potential to generate compensation or public liability claims, public concern and loss of confidence. Therefore, the design and management of plant and comprehensive emergency plans at the company level are crucial.

An incident may arise as a result of:

- Inadequate hardware (e.g. inadequate segregation of incompatible materials)
- Inadequate work practices
- Inadequate emergency planning

Procedures to minimise the likelihood of emergencies, such as accidents, fire, explosion, spills, leaks and natural events (e.g. storms or floods), include:

- Adequate building and facility design, including access for the emergency services, bund walls where appropriate, readily available safety equipment and water and sumps for firewater
- Adequate security
- Correct handling, storage and segregation
- Provision of safety equipment such as smoke/fire/gas detectors, extinguishers, alarms, absorbents and personal protection
- Special storage for explosives and flammables
- Secure storage for radioactives, poisons and infectious agents
- Correct procedures for the use of chemicals, including decanting and re-labelling from larger containers
- Correct procedures for the disposal of chemicals, their containers or contaminated materials

Waste Minimisation and Disposal

Chemical wastes for disposal are subject to the same hazardous chemicals legislation that apply to other categories of chemicals, and should be classified and labelled in accordance with such legislation. Further, hazardous chemical wastes should be classified and labelled in accordance with relevant systems for hazardous wastes. All chemical wastes, including unused chemicals, chemical-containing wastes and emptied containers should be disposed of in a safe and approved way. Ideally, this should form part of the organisations' waste disposal system, which should use the hierarchy of wastes in decreasing level of potential environmental damage, to:

1. Reduce at source
2. Reduce, reuse, or recycle organisational wastes
3. Treat wastes, or
4. Properly dispose to landfill, atmospheric emission, sewerage or trade waste

Record Keeping

In all cases, records should be kept of activities in controlling chemical exposures. Records should be made available to all persons subject to the content of the record. Systems should also be available for archiving and security of records. Where records refer to specific workers by name, they should be subject to privacy requirements and kept for at least 30 years.

Management System Review

Once a management system is in place, it needs to be monitored to ensure it is working, and reviewed to ensure it is effective. There are a range of techniques to monitor and review the management system, including inspections and audits.

Summary

Chemicals are widely used in organisations. While some chemicals are not hazardous, many are. Some present hazards after single exposures, some require repeated exposures to reach a toxic level. Some chemicals have toxic properties, others have physico-chemical properties that present danger to health, property and the environment.

Chemical-induced injury and disease and chemical-induced damage to the environment remain significant problems for employees and industry. The responses of organisational managers to the problems of chemical hazards vary dramatically. These responses are made more complex by the need to comply with a bewildering range of legislation and standards that may seem confusing and contradictory. Accurate and timely information is critical to an effective system. A number of national and international initiatives have recommended the development of conventions, regulations and codes of practice to attempt to deal with the problems of chemical safety at work.

An effective chemical safety management system includes:

- Obtaining commitment from senior management
- Instituting consultative mechanisms
- Developing a chemical safety policy and management system
- Identifying the program components of the chemical safety management system
- Resourcing, implementing and reviewing the programs
- Integrating the chemical safety management system into the organisation's strategic plan

To be effective, chemical safety activities must be integrated into the management functions of planning, organising, leading, coordinating, controlling, directing and evaluating organisational systems.

The contemporary risk management approach of consult, identify, assess, control and review offers a way of dealing with hazardous chemicals in organisations. Processes of handling and using chemicals that form part of everyday organisational activities can be developed in such a way that they do not have adverse impacts on safety, health or the environment. These include good chemical safety management structures such as safe purchasing, inventory control, hazard communication, competency training, risk assessment and systems for the control of chemical risks, storage, organisational environmental monitoring, health surveillance, systems for emergencies, disposal and record keeping.

Such a risk management system for hazardous chemicals will help in providing and maintaining an organisation that is safe and without reasonably foreseeable risks to safety, health and the environment.

References

1. NTC (2007) Australian dangerous goods code, 7th edn. National Transport Council, AGPS, Canberra
2. Safe Work Australia (2005) Hazardous substances information system. Safe Work Australia, Canberra
3. TGA (2009) Standard for the uniform scheduling of drugs and poisons no 25. Therapeutic Goods Administration, Commonwealth Department of Health and Ageing, AGPS, Canberra
4. UNEP (2000) Methodological guide for the undertaking of national inventories of hazardous wastes within the framework of the Basel convention. United Nations Environment Program, Geneva
5. Winder C, Azzi R, Wagner D (2005) The development of the globally harmonised system (GHS) of classification and labelling of hazardous chemicals. J Hazard Mater 125:29–44
6. UNECE (2005) Globally harmonized system of classification and labelling of chemical (GHS). United Nations Economic Commission for Europe, Geneva
7. Winder C (1995) Best practice in workplace hazardous substances management. Qual Assur 4:211–225
8. Winder C, Barter J (2004) Chemicals, workplace and law. In: Winder C, Barter J (eds) Occupational toxicology, 2nd edn. CRC, Boca Raton, FL, pp 492–521; Chap. 20
9. Australia S (2009) AS/NZS ISO 31000 Risk management—principles and guidelines. Standards Australia, Sydney
10. Winder C (1999) Applying risk management approaches to chemical hazards. J Occup Health Safety—Aust NZ 15:161–169
11. Australia S (2001) AS/NZS 4801 Occupational health and safety management. Standards Australia, Sydney
12. Australia S (2004) AS/NZS ISO 14000 Environmental management systems. Standards Australia, Sydney
13. Taouk M, Lasswell P, Winder C (2001) Workplace risk assessment: a practical approach to safety management. J Occup Health Safety—Aust NZ 17:555–566

Toxicology of Water

Xiaowei Zhang, Steve Wiseman, and John Giesy

Abstract To protect the quality of water from toxic pollutants for the health of humans and the environment, two approaches are generally applied in the field of toxicology to predict the effects of pollutants and to monitor the toxic pollutants in water. Here we provide our perspective on state-of-the-art methods to develop water quality criteria and the use of molecular techniques for monitoring water quality. Emphasized is the recent development and application of cell-based assays and small fish model in toxicology research of water.

Keywords Alternative methods for toxicity testing · Bioanalytical method · Bioassay · Cell-based assay · HPG axis · *In vitro* bioassay · Small fish model · Species sensitivity distribution · Water quality criteria

X. Zhang (✉)
State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing 210089, China
e-mail: howard50003250@yahoo.com

S. Wiseman
Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan, Canada
e-mail: steve.wiseman@usask.ca

J. Giesy
Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan, Canada
Department of Veterinary Biomedical Sciences, University of Saskatchewan, Saskatoon, Saskatchewan, Canada
Department of Biology and Chemistry, City University of Hong Kong, Kowloon, Hong Kong, China
e-mail: John.Giesy@usak.ca

A. Luch (ed.), *Molecular, Clinical and Environmental Toxicology*,
Experientia Supplementum 101, DOI 10.1007/978-3-7643-8340-4_2,

Prediction of the Effects of Pollutants: Development of Water Quality Criteria for the Protection of Aquatic Organisms and Humans

The goal of establishing water quality criteria (WQC) for the protection of aquatic life is the protection of the structure and function of the ecosystem from long-term exposures. The methods to conduct these assessments vary among jurisdictions but in general have the same elements and suffer from the same sorts of limitations and uncertainties. The WQC are often structured to protect organisms that might accumulate sufficient exposure through trophic transfer as well as from direct exposure of aquatic organisms. For instance, the WQC should protect animals such as predatory fish, birds, and humans that might eat aquatic organisms such as plankton that would be expected to come to a steady-state distribution with contaminants in water from direct exposures. This can be done through the application of bioaccumulation factors (BAFs) for uptake from water into organisms and biomagnification factors (BMFs) for accumulation through the diet via trophic transfer. These two pathways of exposure are often combined into overall bioaccumulation factors (BAFs). Due to bioaccumulation, the WQC to protect predatory animals from the effects of bioaccumulative compounds (with log $K_{ow} \geq 3.0$), depending on relative sensitivities, are likely to be less than that to protect aquatic animals from direct exposures. Thus, either one comprehensive WQC value can be derived to be protective of both situations or, as is done by some jurisdictions, two separate WQC values could be derived.

Water Quality Criteria for Protection of Aquatic Ecosystems

In some cases, it might be important to protect specific species which might exhibit unique sensitivities or might be of special functional, cultural, or economic importance. In that case, testing of those species might be appropriate. But the many types of organisms that might be critical to the functioning of the ecosystem fall into many different families and orders which might have varying sensitivities to the compounds of interest, and it would be impossible to test all of the individual species. Thus, to be protective, the WQC must take into consideration this variation in sensitivities. There are several approaches to address this issue. One is to select model or sentinel species that, based on experience, have proven to be relatively more sensitive to toxicants. Then, "an application," "an assessment," or "a safety" factor can be applied to increase the probability of the WQC being sufficiently protective. There is some information that has been developed that gives some guidance as to the level of uncertainty factors to be applied, depending on how much information is available and what uncertainties remain (Table 1). For instance, if little data are available or the data are only for acute exposures, then a larger safety factor might be warranted than if a great deal of information is available from chronic exposures, especially with species that are generally known to be sensitive.

Table 1 Comparison of assessment factors in current use (adapted from [1])

Regulatory authority	Dataset[a]	Assessment factor	Notes[b]
Canada[c]	Chronic LOEC	10	3 Species of fish, 2 invertebrates, and algae (or freshwater vascular plant)
Australia	Lowest of ≥5 species chronic NOECs	10	If necessary, NOECs are estimated from other data as follows: MATC/2 LOEC/2.5 LC(EC)50/5
	Lowest of ≥5 species acute LC(EC)50	100 or 10× acute-to-chronic ratio	Applied to the lowest LC(EC)50 value
EU (freshwater)	Acute LC(EC)50	1,000	
	Chronic NOEC	10–100	
EU (marine)	Acute LC(EC)50	1,000–10,000	
	Chronic NOEC	10–1,000	
OECD	Chronic NOEC	10–100	

[a]LOEC = lowest observed effect concentration; NOEC = no observed effect concentration; LC(EC)50 = median lethal or effect concentration
[b]MATC = maximum acceptable toxicant concentration = (NOEC + LOEC)/2
[c]CCME (Canadian Council of Ministers of the Environment) 2007 (draft new protocol)

Water Quality Zones

Some jurisdictions have multiple WQC, depending on the classification of waters, or to what use they are to be put. For instance, a waterway might be classified to be used only for industrial or transportation purposes. In such cases, the WQC might be different if the water is classified for recreational use or for aquaculture production. Even other areas might be defined to be kept as pristine areas where the magnitude of a potential stressor would not be allowed to vary from what has been determined to be natural ranges or at least within ranges appropriate to protect very sensitive components of the ecosystem, such as endemic and/or endangered species.

Toxicity Data Requirements

In the absence of toxicity information for compounds of interest, there are several possible ways to predict the potential toxicity. Some of these methods require the collection of actual toxicity information, while others rely on existing information to make extrapolations. For instance, the toxicity of a chemical of concern can be predicted from information on the toxicity to other species. The use of species sensitivity distributions (SSDs) allows for estimation of the probability of protecting a particular proportion of a population. In some situations, the toxicity of a chemical can be inferred from the toxicity of structurally similar compounds. This approach, referred to as the quantitative structure activity relationship (QSAR), can use

relationships, referred to as linear free energy relationships (LFERs), to predict the toxicity of a chemical of concern from information on the toxicity of similar compounds. Similarly, the potential of chemicals to cause certain types of effects can be inferred from similar relationships where the particular response is predicted from structural and or functional properties of compounds. Thus, if the structure of a chemical is known, some inferences about its toxicity can be predicted. Also, it might be possible to use information collected on freshwater organisms if it is for the protection of marine ecosystems [2]. However, there is no generalization that can be made on whether marine organisms are more or less sensitive to particular contaminants.

Sometimes, partial information is available for a compound or species of interest. For instance, acute toxicity might be available for several species such that a WQC that would be expected to be protective of most species could be predicted from an appropriate acute-to-chronic ratio (ACR) based on the class of chemical. The US Environmental Protection Agency (US-EPA) has developed an acute-to-chronic estimation (ACE v 2.0) model based on time–concentration–effect models (ACE) [3] that allows for this sort of prediction to be made for freshwater species. The software developed uses three different methods to make predictions. These include (1) accelerated life testing (ALT), (2) multifactor probit analysis (MPA), and (3) two-stage linear regression analysis (LRA). Of the three, the US-EPA suggests that the method of choice is ALT.

If toxicity information is available, there are basically two types of models that are used: time to effect or concentration to cause a defined effect in a specified time. In one, the duration of exposure is set at a fixed period of time (e.g., 24 h, 7 days, or 21 days), and the magnitude of the parameter or concentration of chemical required to elicit a particular level of effect is determined. In this type of study, the endpoint might be a quantal effect such as lethality or it might be a continuous variable such as growth or reproduction. The level of effect, such as the median effect concentration (EC50), which would be the concentration to cause adverse effect to 50% of the population, would be selected. The level of effect can be chosen as appropriate for any level of protection desired. Then by analyzing data from a range of concentrations, a function could be fit to the data to interpolate the desired effect level for a specified duration of exposure. In the second approach, the concentration is fixed and the duration of time to cause the specified level of effect is determined by observing the responses of organisms exposed to different concentrations for different periods of time. It is suggested that both methods have advantages, that both types of data be collected, and that toxicity curves, incorporating both the duration and magnitude of exposure, be developed [4, 5]. In this way, a reciprocity relationship can be developed that allows determination of both the incipient (threshold) duration and threshold concentration for effect. In fact, by developing the toxicity curve, the greatest amount of data can be extracted from the dose–response relationships.

Data Collection

Toxicity data can be obtained from searches of the most updated US-EPA ECOTOXicology database (ECOTOX). ECOTOX integrates three previously independent databases—AQUIRE, PHYTOTOX, and TERRETOX—into a unique system, which includes toxicity data derived predominately from the peer-reviewed literature, for aquatic life, terrestrial plants, and terrestrial wildlife, respectively. The current information in the ECOTOX (Version 4) database now contains a total of 285,798 records, with the majority of the literature reviewed from 1972 to 2008. The number of references, species, and chemicals included in these data are 18,831, 4,999, and 7,630, respectively. The quality of the toxicity data obtained from ECOTOX (2009) has been assessed by the US-EPA to determine how the toxicity data were generated using a number of criteria. Data not meeting these criteria were excluded from the ECOTOX databases.

Once the quality of the toxicity data has been determined, the aqueous solubility of the compounds needs be considered. There can be considerable error in determining aqueous solubility particularly for hydrophobic chemicals. Therefore, data that reported toxic effects at concentrations greater than twice the aqueous solubility should be removed. Also, the toxicity data will be screened in terms of the endpoints that they measured. Only toxicity data that measured lethality, growth, immobilization, photosynthesis, and reproduction should be considered as being environmentally relevant [6]. In particular, biochemical and behavioral endpoints should be excluded because of their doubtful ecological significance [7, 8].

In order to ascertain the likelihood of differential chemical sensitivity among different groups of species, the dataset should consist of acceptable acute (or chronic) test results of the key taxonomic groups of aquatic species (Table 2). To be protective of animals from long-term exposures to toxicants, the WQC should be based on chronic toxicity. However, it is also useful to have a WQC for short-term or spill type situations. This value, termed the maximum acceptable

Table 2 Data requirements for the modified Great Lakes Initiative (GLI) Tiered Method proposed for the derivation of water quality criteria (WQC) for protecting saltwater ecosystems such as Hong Kong coastal marine waters

Data type	Required data for saltwater (SW) systems
A. Results of acceptable acute (or chronic) tests for:	1. One SW fish 2. One SW algae/cyanobacterium/fungus 3. One SW mollusk 4. One SW crustacean 5. One SW polychaete 6. One other SW invertebrate (e.g., echinoderm)
B. Acute-chronic ratios with data for at least:	1. One SW fish 2. One SW invertebrate 3. One other SW or freshwater species (if data not available, then the other two may be freshwater species)
C. Data for at least:	1. One SW algae or vascular plant (if data not available, then freshwater algae or vascular plant)

toxicant concentration (MATC) is based on the short-term or acute toxicity information.

Since there is often more acute toxicity information available, usually acute lethality data are used to predict the concentration of a chemical to which animals can be exposed for longer periods of time by use of the ACR. This ratio is used to estimate allowable chronic exposure concentrations for other species for which little chronic toxicity data exist. The ACR is derived by dividing a compound's acute LC_{50} value by the chronic nonobservable effect concentration (NOEC) for the same species. One limitation to this approach is that biological endpoints/responses are sometimes not comparable between acute and chronic studies. Acute studies involve lethality as an endpoint, while a chronic MATC is often derived from an endpoint other than lethality (growth, reproductive ability, *etc*.). Although the mode of action for lethality is assumed to be the same under acute and chronic exposures, the mode of action may not be the same for different toxicity endpoints. For a variety of organisms and chemicals, the ACR has been found to be approximately 10 [9]. This value is supported by QSARs for some chemical groups, in which acute and chronic regressions are separated by about an order of magnitude [10–12].

Derivation of Water Quality Criteria

Once the toxicity information has been assembled, there are basically two approaches that can be taken. The first approach, termed the assessment factor (AF or application factor) method, which is used by the USA, EU, Australia, and South Africa, is where data on the NOEC from toxicity tests are divided by an arbitrary factor (10 to 1,000) to account for uncertainties, such as among-species differences in toxicity, a lack of data on some types of species, or tests of sufficient duration or of the most sensitive life stages. The AF is essentially a safety factor meant to protect species and/or ecosystem integrity instead of predicting potential for effects. The other approach, which is applied when more information is available, is the statistical extrapolation approach (i.e., probabilistic approach). In this approach, statistical approaches are used to relate the concentration and duration of exposure to the level of effect. Both methods are based on acute or chronic toxicity test results with either surrogate or local species.

Species Sensitivity Distribution Approach

Probabilistic approaches have been used to describe the among-species sensitivity [13] (Fig. 1). In the SSD approach, the probability of a threshold for effect for either acute or chronic exposures being exceeded is developed. For instance, in this approach, the probability of a particular proportion of individuals of a particular proportion of species can be estimated. This approach requires data on the dose–response relationships for a relatively large number of species. As an

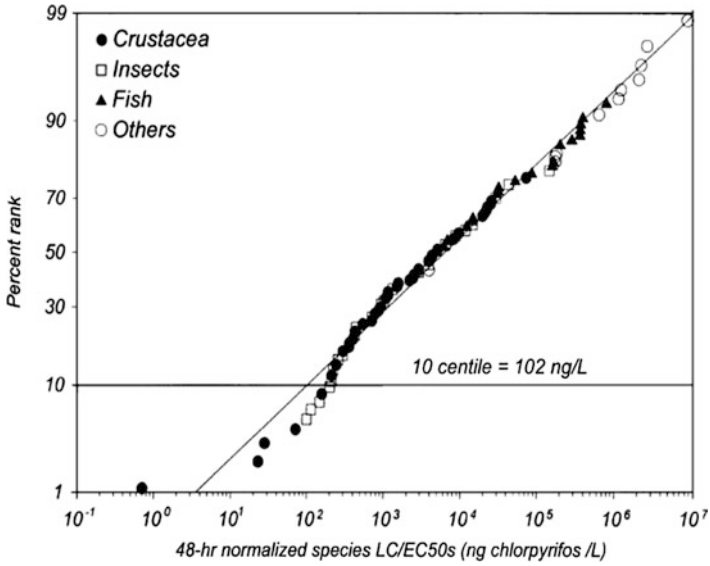


Fig. 1 An example showing the species sensitivity distribution of the organophosphorus insecticide chlorpyrifos for freshwater aquatic species (adapted from [13])

example, if data for only two species were available, each species would represent 50% of the frequency distribution and resolution or prediction would be poor. As a rule of thumb, it is suggested that to use the SSD approach, data for a minimum of 20 species should be available. In this case, each species would represent 5% of the total, and the resolution of the predictive power of the analysis would be 5%. The SSD approach assumes that the species that have been tested represent a random selection of all the possible sensitivities. If, for instance, the data available were for similar species with similar sensitivities that did not represent the entire range of possible sensitivities, then the predicted probabilities would not be accurate.

In addition to determining the probability of effect, one can also determine the probability that a particular exposure concentration, associated with a defined probability of effect can be determined (Figs. 2 and 3) [5, 13]. In this analysis, a relatively large number of measurements of the concentration of a contaminant are required. Therefore, the SSD approach will enable probabilistic ecological risk assessments of toxic chemicals in the marine environment.

The SSD approach is the preferred method by many developed countries, such as the Netherlands, EU, and Australia, although a variety of models to fit the SSD have been used. These models include parametric models (log-normal and log-logistic models), semiparametric models (i.e., bootstrap regressions) and nonparametric bootstrap model as well as Burr type III models. Different models can generate very different WQC values at the same level of protection (e.g., 95%) [14]. Thus, the best model fit should be carefully selected to generate the most accurate WQC value. For instance, such comparisons can be easily made using the Burr distribution software developed by CSIRO of Australian Government, which allows

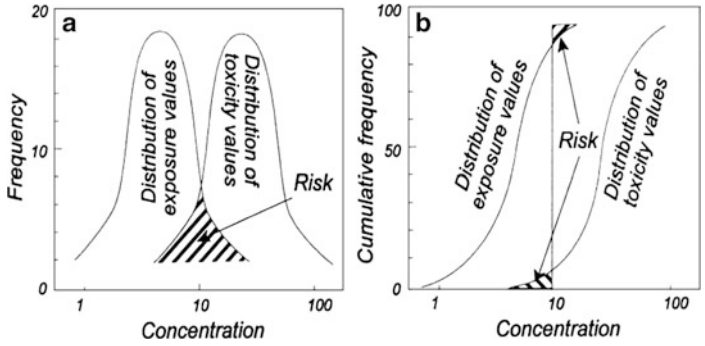


Fig. 2 Relationship between effect and exposure concentration distributions expressed as log-normal distributions (**a**) and cumulative log-normal distributions (**b**) (adapted from [13]). The effect concentration distribution (i.e., distribution of toxicity values) is represented by a species sensitivity distribution

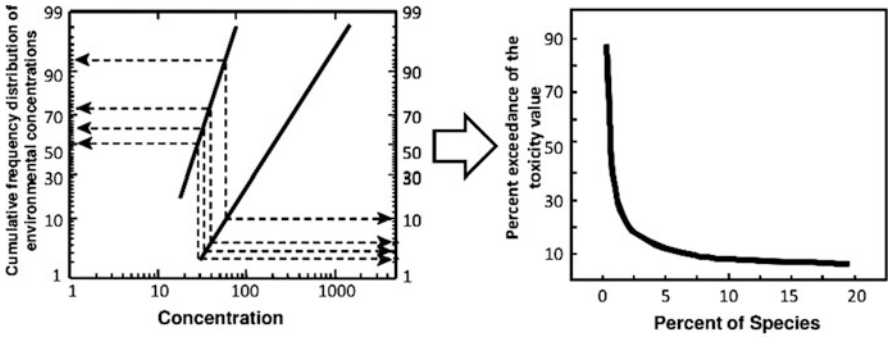


Fig. 3 Illustration of the derivation of a joint probability curve/exceedence profile from exposure and toxicity probability functions (adapted from [5, 13]). The dashed line and solid line on the left subfigure represent the linearized probability distributions of exposure and toxicology data

simultaneous comparison of various model fits for a SSD and is freely available for download from the CSIRO website (http://www.cmis.csiro.au/envir/burrlioz/).

The SSD probabilistic approach is useful if there are sufficient data and the implicit assumptions of the SSD method are met. In particular, the SSD method is useful in determining the types of organisms that are the most sensitive. It is also data intensive. To generate useful predictions, toxicity information is required for a large number of species and those species need to be distributed among representatives of different groups that might have different sensitivities or might have different exposures. Also, because it is a probabilistic approach, there are no values for zero or 100%. This is important because when representing the procedures used to derive WQC to the public, it is difficult to portray the risk. In general, the public does not want to accept any risk, but by definition, when applying the SSD approach, some proportion of the individuals of some proportion of the species will be affected some

proportion of the time. Our experience has led us to understand that exposures in the environment are generally less than those under controlled laboratory testing and that there is a certain amount of redundancy in the functions of environments that leads to a certain level of ecosystem resiliency. For this reason, setting the WQC based on the 5th centile is probably protective of most individuals of most species most of the time. In fact, when exposures set at the 5th centile have been compared to the results of multispecies mesocosm studies, the 5th centile has not resulted in measurable responses in populations or ecosystem function. But to the public, it appears that the regulations will allow for 5% of all species to be adversely affected. Thus, from a policy perspective, it becomes difficult to explain, let alone defend regulations based on this approach. In the following section, we present an analysis comparing the results of the SSD and GLI-type approaches. We present the relative protection afforded by each of the approaches.

Great Lakes Initiative Approach

One method developed by the US-EPA for use in developing WQC for the Great Lakes [5] is a semiprobabilistic approach that specified the types of data that are required and depending on the quality and quantity of toxicity data available assign assessment factors to correct the data to account for these uncertainties. This approach, referred to as the Great Lakes Initiative (GLI), allows development of WQC for the protection of aquatic organisms. In addition, BCFs and BMFs can be used to derive BAFs that can be applied to infer if the WQC are sufficient to protect higher trophic level organisms such as humans that might eat aquatic organisms.

While these are large bodies of water, the Great Lakes are freshwater. Thus, the WQC values that have been developed based on this method would not be directly applicable to other aquatic environment WQC, such as marine. However, it is suggested that the method is appropriate to be used to generate toxicity information for local organisms [5]. This method is a semiprobabilistic approach that specifies a required minimum dataset. By specifying data requirements for different classes, families, and genera, the method maximizes the potential for including a species of a type that would be expected to be sensitive to the stressor of concern. For instance, because a plant species (e.g., microalgae or macroalgae) is required in the dataset, there would be a species sensitive to the effects of herbicides. The GLI methodology is useful, because it allows for different levels of completeness in the datasets and specifies uncertainty factors to apply when the datasets are deemed to be insufficient. Two levels of WQC can be derived using this methodology. The first or Tier I value is calculated when all of the necessary information is available and is the value for which there is greater confidence. If less data are available or the data are of lesser quality, then a Tier II value can be calculated by the use of assessment factors. In this way, there is a built-in mechanism to facilitate collection of additional data because the additional data reduce uncertainty and in doing so can result in greater values for the WQC. This gives the regulatory community

incentive to provide the resources needed to conduct the testing. The method also has the advantage that it takes into account multiple datasets. It is suggested that a listing of specified species and or classes and families appropriate to capturing likely sensitive local species be developed to make a GLI-like approach workable for the aquatic system of concern. Finally, the GLI-type approach considers both acute and chronic toxicity information and allows prediction of one from the other, by use of an ACR, which might be derived as a species-specific factor or derived from among stressor comparisons.

In addition to the development of WQC for the protection of aquatic life, the GLI presents methodologies to develop WQC for the protection of higher trophic levels from potential effects of bioaccumulated chemicals, and biota–sediment accumulation factors can be used to calculate sediment quality objectives to protect both aquatic life and higher trophic levels. We favor the use of this GLI-like approach because it is flexible and a probabilistic approach. Because specific types of organisms are required for the analysis, it does not suffer from the limitations of as SSD approach alone. In addition, the GLI approach allows for the calculation of WQC for compounds with differing amounts of toxicity data. The GLI approach takes into account the amount and quality of data available and uses AFs to correct the WQC. The use of the GLI-type approach also allows the risk assessor the opportunity to review the data. In doing so, the risk assessor can assess the quantity and quality of data available and determine which groups would be most at risk and apply safety factors where necessary to protect ecologically or economically valuable species or maintain overall ecosystem structure and/or ecological functions. The GLI approach also allows the use of multiple testing with the same species without weighting each test as would be done if each test were given as a separate test result or losing an estimate of the variability in the data as it would be in a strict SSD. This approach rewards having more data and encourages the development of additional data to reduce uncertainty. Finally, the flexibility of the approach allows risk managers to apply more or less stringent protection criteria in a risk–benefit approach.

The GLI-type approach avoids some of the limitations of the standard SSD approach. In the SSD approach, there are some implicit assumptions that apply. It is assumed that the species for which toxicity data are available are a fair representation of the population of possible sensitivities. For instance, an SSD can be developed from data for 50 species, and a probability of effect, such as the 5th centile, can be derived. However, if all of these species were closely related, they could not represent a true measure of the entire range of sensitivities. For this reason, a straight SSD approach can give a false sense of accuracy and precision.

Issues Related to Chemical Mixtures

Chemicals of environmental concern often occur as a mixture such as polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs), both of

which are currently listed as priority toxic chemicals by some jurisdictions. The presence of different compounds in a mixture presents a challenge to WQC derivation. For instance, different isomers and congeners of PCBs are known to have different physicochemical properties (e.g., various persistency) and, hence, different toxicities (i.e., different potencies) to animals. Due to variation of pollution sources and weathering, the composition of PCBs can vary considerably in water samples among different sites. Therefore, this raises a fundamental question whether or not the WQC derived for a mixture, like total PCBs, is protective of marine organisms and human. At present, there are two main approaches for derivation of WQC for a mixture such as PCBs, namely, the total mixture method (identical to that of single compound) and the toxicity equivalence quotient (TEQ) method. Therefore, our review will also address the relative merits of these two approaches for developing WQC and conducting risk assessments.

Here we present total PCBs as an example. PCBs are members of the group of halogenated aromatic hydrocarbons and consist of 209 isomers and congeners with different numbers and positions of chlorine atoms substituted on the biphenyl moiety. Individual PCB congeners exhibit different physicochemical properties which results in different profiles for environmental distribution and toxicity. PCBs have low water solubility, which decreases with increasing degree of chlorination. For example, the water solubilities of monochlorobiphenyl congeners are in the range of 1–5 g/L but that of decachlorobiphenyl is only 0.015 mg/L [15].

The advantages of total PCB-based WQC derivation and/or risk assessment include its simplicity by being used as a conventional method. This approach also incorporates risks due to metabolites and interactions among congeners. The ability of animals to metabolize PCBs does not necessarily imply that the metabolites can be excreted and therefore that risk can be minimal. The potential adverse effects of PCBs on marine organisms are dependent on several factors including the overall concentrations of PCBs to which they are exposed and the relative toxic potencies of the individual congeners present in the mixture and their interactive effects. Due to the limitations of the total PCB-based approach in risk assessment, application of congener specific risk assessment methods has been suggested.

Alternatively, the TEQ approach allows the expression of the toxic potential of a complex mixture of individual congeners as one integrated parameter, the toxic equivalency value, in which the toxic potency of the mixture corresponds to the potency of the most toxic congener of PCBs, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). If relative potencies can be derived for PCB congeners for a few endpoints and species that are found to be intercorrelated and if congeners can be established to have the same rank order among endpoints and species, the relative potencies can be used to develop a toxic equivalency factor (TEF) for each congener. As an example of the technique, if the ED50 values for immunosuppressive activity of TCDD and 1,2,3,7,8-penta-CDD were 1.0 and 2.0 μg/kg, respectively, then the TEF for the latter compound would be the ratio of $ED50_{(TCDD)}/ED50_{(1,2,3,7,8\text{-pentaCDD})}$, or 0.5. TEF values have been determined for several different aryl hydrocarbon receptor (AhR)-mediated responses. However, for every PCB congener tested, the TEF values are response and species dependent [16]. As an example, TEFs for

TCDD obtained from *in vivo* and *in vitro* studies varied from 0.17 to 0.016 and 0.43 to 0.006, respectively [17]. Regulatory agencies have chosen consensus TEF values for individual congeners. Selection criteria have been based on the importance of data obtained for specific responses (e.g., carcinogenicity, reproductive, and developmental toxicity).

The TEF approach was first utilized to assess the risks associated with air emissions of polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) formed during high-temperature incineration of industrial and municipal waste [18, 19]. Subsequently, the US-EPA proposed interim criteria for estimating risks associated with mixtures of PCDDs and PCDFs for other media as well [20]. Several international agencies have also adopted the TEF approach for the risk assessment of PCDDs and PCDFs [21–24]. The mechanistic considerations for the development of TEFs for the risk assessment of PCBs have been described elsewhere [17, 25]. TEF values have been proposed by the WHO for mammals, birds, and fish [26, 27]. We will briefly review the development of TEFs using mammalian models [17] and the recent progress in studies relating to fish- and bird-specific TEFs for PCBs. Such techniques may be extended to other mixture chemicals.

Approach for Deriving Chemical Water Quality Criteria for the Protection of Wildlife

Because the water not only supports numerous human activities and provides habitat for aquatic organisms, but also sustains viable mammalian and avian wildlife populations, chemical WQC need to be derived for the protection of populations of these predatory animals. The water quality objectives for mammalian and avian wildlife are surface water concentrations of toxicants that will cause no significant reduction in the viability or usefulness (in a commercial or recreational sense) of a population of exposed animals utilizing target waters as a drinking and/or foraging source over several generations. The application of the proposed approach requires the acquisition of BCFs and BAFs (trophic transfer coefficients) as well as the development of toxicity reference values (TRVs) for each of the priority pollutants. Furthermore, appropriate application factors need to be derived depending on the quality and quantity of data available. In general, the number of studies available for the higher trophic level species is less, and some studies do not consider an entire life cycle or the most sensitive and ecologically relevant measurement endpoints. For this reason, safety factors are normally applied. The most appropriate set of safety factors are those given in the GLI methodology (cf. above). The procedure for selecting the overall safety factors is well described and results in transparent safety factors so that the risk assessor can understand where the uncertainties lie and the magnitude of uncertainty factors that have been applied.

Calculation of Tier I Wildlife Objectives and Tier II Wildlife Values

The equation used to calculate wildlife values (WV) has both an effect and an exposure component. The effect component is defined as the test dose (TD) which is either a lowest observed adverse effect level (LOAEL) or a no observable adverse effect level (NOAEL) for milligrams of substance per kilogram body weight per day (mg/kg BW/day). The exposure routes considered in this derivation are food and water ingestion, and because the intake level is dependent on organism size, it is scaled to body weight. The total toxicant intake through these exposure routes is determined and then set equal to the TD (1) and (2):

$$\text{Toxicant intake through drinking water} = (\text{WV} \times W)/\text{Wt} \tag{1}$$

$$\text{Toxicant intake through food} = [\text{WV} \times \Sigma(F_{\text{TLi}} \times \text{BAF}_{\text{TLi}})]/\text{Wt} \tag{2}$$

where: WV = Species-specific wildlife value in milligrams of substance per liter (mg/L). W = Average daily volume of water consumed in liters per day (L/day) by the representative species. F_{TLi} = Average daily amount of food consumed from trophic level in kilograms per day (kg/day) by the representative species. BAF_{TLi} = Bioaccumulation factor for wildlife food in trophic level in liters per kilogram (L/kg). For consumption of piscivorous birds by other birds, the BAF is derived by multiplying the trophic level three BAF for fish by a BMF for biomagnification of the chemical from fish to birds that consume these fish. Wt = Average weight in kilograms (kg) for the representative species.

Equations (1) and (2) are combined to yield (3):

$$\text{TD} > (\text{WV} \times W)/\text{Wt} + [\text{WV} \times \Sigma(F_{\text{TLi}} \times \text{BAF}_{\text{TLi}})]/\text{Wt} \tag{3}$$

where TD = Test dose in milligrams of substance per kilogram body weight per day (mg/kg BW/day) for the test species (either a NOAEL or LOAEL derived from mammalian or avian toxicity studies).

To account for differences in toxicity among species and uncertainties in LOAEL to NOAEL extrapolations and subchronic to chronic extrapolations, the TD is divided by three uncertainty factors: UF_{A}, UF_{s}, and UF_{L},

where:

UF_{A} = Uncertainty factor for extrapolating toxicity data across species (unitless). A species-specific uncertainty factor shall be selected for each representative species.

UF_{s} = Uncertainty factor for extrapolating from subchronic to chronic exposures (unitless).

UF_{L} = Uncertainty factor for LOAEL to NOAEL extrapolations (unitless).

The final equation of the WV therefore is (4):

$$WV = \frac{\frac{TD}{UF_A \times UF_s \times UF_L} \times Wt}{W + \sum [FT_{Li} \times BAF_{TLi}]} \quad (4)$$

Derivation of the Final Tier I Wildlife Objective

The wildlife values specific for each taxonomic class are derived by taking the geometric mean of the wildlife values across all of the representative species within each taxonomic class (5):

$$WV_{(\text{taxonomic class})} = \text{Exp}\left[\Sigma \ \ln WV_{(\text{representative species})}/n\right] \quad (5)$$

where n = The number of representative species in a given taxonomic class for which species-specific wildlife values were calculated.

The water quality objective is then set equal to the lower of the two taxonomic class-specific wildlife values.

Derivation of a Tier II Wildlife Value

The equation to derive a Tier II wildlife value is the same as that presented above to derive the taxonomic class-specific Tier I wildlife values which are then used to determine the Tier I wildlife criterion. One of the major differences in the derivation of a Tier I wildlife value and a Tier II wildlife value is that for a Tier I wildlife objective, a taxonomic class-specific wildlife value is derived for both taxonomic classes, such as *Aves* and *Mammalia*, while a Tier II wildlife value can be determined when a taxonomic class-specific wildlife value is available for only one taxonomic class.

Monitoring of Toxic Chemicals

Development of WQC provides criteria for efficient monitoring and management of a number of prioritized toxicants in aquatic environments by relying on a large amount of toxicity data. There are thousands of chemicals including industrial and agricultural chemicals that could be spilled or discharged into aquatic systems and lead to human and wildlife exposures; however, most of these chemicals have very limited toxicity information [28]. This is primarily due to the high cost and time required to conduct conventional toxicity testing with many species.

Also, animals are more often exposed to mixtures of chemicals rather than a single chemical. Therefore, what is needed are alternative toxicity testing methods that can be used to efficiently evaluate the adverse effects of mixtures of environmental chemicals or effluents. The recent development of cell-based *in vitro* bioassays and small fish models in toxicological research of water is reviewed here.

Cell-Based In Vitro Bioassays

Cells used in *in vitro* bioassays can be permanently established eukaryotic cell cultures that are used to measure specific toxic effects or to detect the presence of specific classes of toxic chemicals in samples related to aquatic systems (water, sediment, suspended matter, biota). Cell-based *in vitro* bioassays can be classified based on whether the cell line is an untransformed wild type or has been genetically modified. Each type of cells has advantages and disadvantages for identifying a specific mechanism of toxic action or exposure to a certain group of chemicals. Cell lines are generally derived from tumor cells due to their proliferative properties that are suppressed in healthy tissues. These cell lines are then immortalized with the aim to maintain their particular properties that enable the detection of chemicals or potential of environmental samples to interact with specific biological pathways. For example, since it expresses all the key steroidogenic enzymes and produces most of the steroid hormones, such as mineralocorticoids (aldosterone), glucocorticoids (cortisol), and sexual hormones (estradiol and testosterone), the human H295R adrenocortical carcinoma cell line has been widely used to assess chemical-induced effects on steroidogenesis [29–33]. Chemical agents that alter production of steroids at the cellular level have the potential to disrupt the endocrine system in living organisms. Examination of the expression of different steroidogenic enzymes provides mechanistic information on the molecular basis of the altered hormone biosynthesis by chemical exposure. In the H295R assay, cells are exposed to different concentrations of a chemical to assess the presence or potential of compounds that modulate steroid hormone synthesis. Biological effects can then be measured at different organizational levels such as gene expression, enzyme activity, or hormone production as desired [31, 33].

Sometimes, wild-type cell lines are genetically modified to express favorable attributes that allow the detection of certain classes of chemicals. They often use different combinations of endogenously expressed elements and exogenous factors that are artificially introduced into the cell system. For example, the H4IIE-*luc* is a stably transfected cell line used in a transactivation assay to detect dioxin-like chemicals [34]. In this cell line, the aryl hydrocarbon receptor (AhR) and the aryl hydrocarbon receptor nuclear translocator protein (ARNT) are endogenous, but an exogenous dioxin-responsive element (DRE) and the reporter gene (luciferase) were stably transfected into the cells as a construct. Once incorporated into the genome of the cells, the induction of the introduced luciferase gene as measured by light emission is proportional to exposure to AhR agonists. In fact, in this particular

example, the amount of light produced is proportional to the potency of mixtures of AhR agonists and is proportional to the toxicity of the mixture. Similarly, transcriptional assays have been developed for a number of other endpoints such as the characterization of the potential of chemicals to agonistically or antagonistically bind to the estrogen or androgen receptor.

Besides well established mammalian cell lines, there are increasing numbers of nonmammalian cells, particularly those isolated from aquatic species, such as fish and amphibians, applied in the toxicological research of water. For example, a rainbow trout (*Oncorhynchus mykiss*) fibroblastic cell line, RTG-2, has been applied to assess chemical-caused genotoxicity using random amplified polymorphic DNA (RAPDs) analysis [35] or micronuclei (MN) estimations by means of flow cytometry [36]. An embryonic fibroblast-derived cell line XTC-2, which was derived from the African clawed frog (*Xenopus laevis*), was used to investigate exposure to environmentally relevant concentrations of the bactericidal agent triclosan by quantifying effects on thyroid hormone associated gene expression [37]. Recently, a gill epithelial cell line from seawater-adapted tilapia (*Oreochromis niloticus*) has been developed to assess exposure and potential effects of toxicants in marine water [38]. As an example of a genetically modified fish cell line, SJD.1 is a zebra fish (*Danio rerio*) caudal fin cell line transformed with a metallothionein (MT) reporter construct, which can be used as to assess exposure to heavy metals [39].

Application

Cell-based *in vitro* bioassays have been widely used for chemical screening and prioritizing approaches such as effect-directed analysis (EDA) or toxicity identification and evaluation (TIE) and the study of chemical-induced molecular mechanisms of toxicity. Assessment of cytotoxicity, e.g., by means of the MTT or Live/Dead® assay, is used to determine the general toxic potential of chemicals or environmental samples that can kill cells either directly or indirectly through the inhibition of cellular metabolic pathways. Genotoxicity tests are used to examine harmful effects induced by chemicals on genetic materials in cells, such as DNA strand breakage or base oxidation [39, 63]. Furthermore, cell lines have been applied in functional *in vitro* assays. For example, the H4IIE-*luc* reporter gene assay has been used to assess the potency of individual AhR active compounds or the overall potency of complex planar halogenated hydrocarbon (PHH) mixture in environment samples [34, 40, 41]. In these examples, chemical or environmental samples can be ranked by their differences in potency using the same cell system.

In addition to their utilization in research on the interactions of chemicals with certain biological pathways, cell lines can be used as bioanalytical tools in environmental diagnostics such as the above described EDA or TIE approaches. EDA is based on a combination of fractionation procedures, biotesting, and subsequent chemical analyses to aid in the characterization of exposure to pollutants in

complex environmental samples [42, 43]. Particularly, the use of *in vitro* bioassays as part of EDA has been shown to be a powerful tool in support of the exposure characterization step in environmental risk assessments and already is routinely utilized in environmental monitoring programs [44]. The vulnerability or sensitivity of cellular components or pathways to exposure with certain chemicals, such as ligand-induced receptor-mediated responses, renders them useful tools to detect the presence of pollutants in aquatic media such as surface water, sediments, or suspended particulate matter. This is particularly true in environments that are characterized by exposure to chemical mixtures. In such situations, the sole use of classic chemical–analytical techniques is not suitable for characterizing exposure due to extreme cost and limits in the available analytical methodologies for many chemicals, especially as often no *a priori* knowledge of the chemicals present in the sample exists. As a consequence, there is an increasing trend of supplementing chemical analysis with bioanalytical techniques that make use of the specific properties of certain groups of chemicals to interfere with specific biological processes. For example, the gene expression of cytochrome P4501A (CYP1A) in hepatic cells and the luciferase activity in H4IIE-*luc* cells can be used to characterize the exposure to dioxin-like compounds [34]. MVLN cells, in which the luciferase gene is under the control of an estrogen receptor-responsive element, and H295R cells have been used to detect the presence of endocrine-disrupting chemicals in water and sediment samples [45, 46]. Under circumstances where there is little information on the identities of the chemicals in a solution, for example, in an effluent discharged into an aquatic system, nontarget screening approaches using a battery of cell lines with different diagnostic properties (e.g., AhR, endocrine disruptor, genotoxicity) can be employed to aid in the identification of the biologically active components.

Beyond their utility as screening assays, cell lines are useful to identify mechanisms of toxicity because the initiating events and any subsequent interactions of a chemical with an organism occur at the cellular or subcellular level (e.g., alteration of the transcriptome). Mechanistic studies using cell lines include but are not limited to examining chemical-caused oxidative stress, altered cellular signaling pathways, and modulated responses at the genomic, proteomic, and/or metabonomic level. For example, simultaneously examination of chemical-induced effects at transcriptional, enzymatic, and metabolite levels in the H295R cells has been used to characterize the disruption of steroid hormone production by endocrine active chemicals [30, 31, 33]. A recent study demonstrated that the strategy of measuring multiple endpoints is effective to differentiate between chemical-caused direct inhibition of aromatase activity from indirect inhibition (e.g., by altering transcriptional expression) [47].

Recently, with the increasing development of *in vitro* assays and application of high-throughput technologies in toxicity testing, there is a demand for a data-driven and science-based system that can classify chemicals based on toxic mechanisms and prioritize chemicals for animal testing. These new techniques are increasingly being used in priority chemical screening programs such as Tier 1 of the Endocrine Disruptor Screening Program of the US-EPA [48]. During these screening

initiatives, large amounts of multidimensional data (e.g., gene transcripts, proteins, and enzyme products) are collected for various concentrations of each chemical analyzed. Concentration (dose)–response relationships of endpoints at the molecular and cellular level provide essential mechanistic information for the toxicity of each tested chemical. To efficiently manage and interpret these large datasets, a novel computational toxicology program, ToxClust, was developed which can cluster chemicals based on concentration–response data derived with single or multiple endpoints [49].

As an alternative approach to *in vivo* animal testing methods, cell-based *in vitro* assays used in aquatic toxicology have advantages, which include (1) cost-effectiveness, (2) short testing time, (3) representing the primary targets of chemical-induced effects, (4) increasing the number of chemicals to be tested by amenability to high throughput, and (5) obviation of the need for the use of whole animals. In order to completely replace animal model-based testing by cell-based *in vitro* assays, much work is underway to address the limitations such as low metabolic capability. One common approach to circumvent this issue is to introduce a metabolism step by supplementing the assay with S9 fraction or microsome treatment. Other well-known limitations of *in vitro* approaches include their abnormal biology, lack of tissue/organ organization, and limited kinetic and dynamic extrapolation [50]. However, increased reliance on the use of cell-based approaches is expected for toxicology studies. With proper application and data interpretation, cell-based *in vitro* bioassays will play an important role in risk identification and prioritization of chemical testing.

Small Fish Models

There are two major challenges which must be met in order to effectively address the toxicology of water. First, methods to efficiently evaluate the toxicity of the large number of chemicals that could enter aquatic environments must be developed. Second, methods to predict chemical-induced toxicities among large number of species are required. Coinciding with the increased reliance on the use of cell-based approaches for toxicology studies, recent development in small fish models, such as the Japanese medaka (*Oryzias latipes*), zebra fish, and fathead minnow (*Pimephales promelas*), have gained much popularity in toxicity testing and risk assessment. The development of small fish models is attractive not only because these *in vivo* models carry normal metabolic capability, relevant organ/tissue organization, and regular growth states but also because the data generated from *in vivo* studies are ready to inform decisions by risk assessors. Fish acute and chronic tests are key components in ecotoxicity testing for quantitatively evaluating the potential adverse effects of substances and effluent discharges.

Compared to large fish models and other mammalian toxicological models, small body size fish models have advantages favoring toxicity evaluation of a large number of chemicals. First, their small body size and ease of culture (breeding

and cultivating) under common laboratory condition make them useful for studies with reduced cost. Secondly, small fish models share many biological similarities with human and other mammalian species, which make them an ideal testing model to assess chemical-induced neurotoxicity, developmental toxicity, hepatotoxicity, and reproductive toxicity. Third, a plethora of literature regarding the physiological, embryological, genetic, genomic, and toxicological knowledge about these small fishes is readily available [51, 52]. Specially, the available genomic information of these small fish models allow the application of toxicogenomics to evaluate toxicity of different chemicals across the genomes of these fish and extrapolate the observed toxicity in these models to humans and other species.

Medaka Hypothalamic–Pituitary–Gonadal Axis Model

Mechanistic information derived at biochemical and molecular levels could provide essential clues to predict effects among species and chemicals [52–54]. To illustrate the recent development of small fish models in chemical toxicity evaluation, the Japanese medaka was used as an example in the testing and evaluation of endocrine-disrupting chemicals [54]. The hypothalamic–pituitary–gonadal (HPG) axis plays a critical role in the development and regulation of the reproductive system in vertebrates. The significant conservation in the basic aspects of the HPG axis across vertebrates makes the use of small fish species for identifying and assessing the effects of EDCs possible [55]. Recently, a HPG-PCR array system has been developed to study effects of chemicals on the HPG axis of the Japanese medaka. This Japanese medaka HPG-PCR array carries the quantitative performance of SYBR Green-based real-time PCR and the multiple gene profiling capabilities of a microarray when examining expression profiles of 36 genes associated with endocrine pathways in the brain, liver, and gonad (Fig. 4). The key signaling pathways and functional processes within the brain (including hypothalamus and pituitary), gonad, and liver of Japanese medaka were included in the HPG transcriptional model. The selected genes consisted of receptors (including steroid receptors, peptide receptors, and lipid receptors), peptide hormones, steroidogenic enzymes, and other key receptor-responsive genes. A pathway-based approach using modified GenMAPP software was implemented to analyze and visualize concentration- and time-dependent gene expression in the HPG axis of Japanese medaka exposed to environmental chemicals. Furthermore, phenotypic anchoring strategies were applied by intercorrelating the gene expression data with physiological alterations and reproductive performance, including fertility and fecundity observed during exposure [56].

The Japanese medaka HPG-PCR array has potential not only as a screening tool of potential endocrine-disrupting chemicals but also in elucidating mechanisms of action. For example, both the anabolic androgen 17β-trenbolone (TRB) and the aromatase inhibitor fadrozole (FAD) can cause decreased plasma concentrations of 17β-estradiol (E2) and reduce fecundity of fish. The mechanisms of the reduced

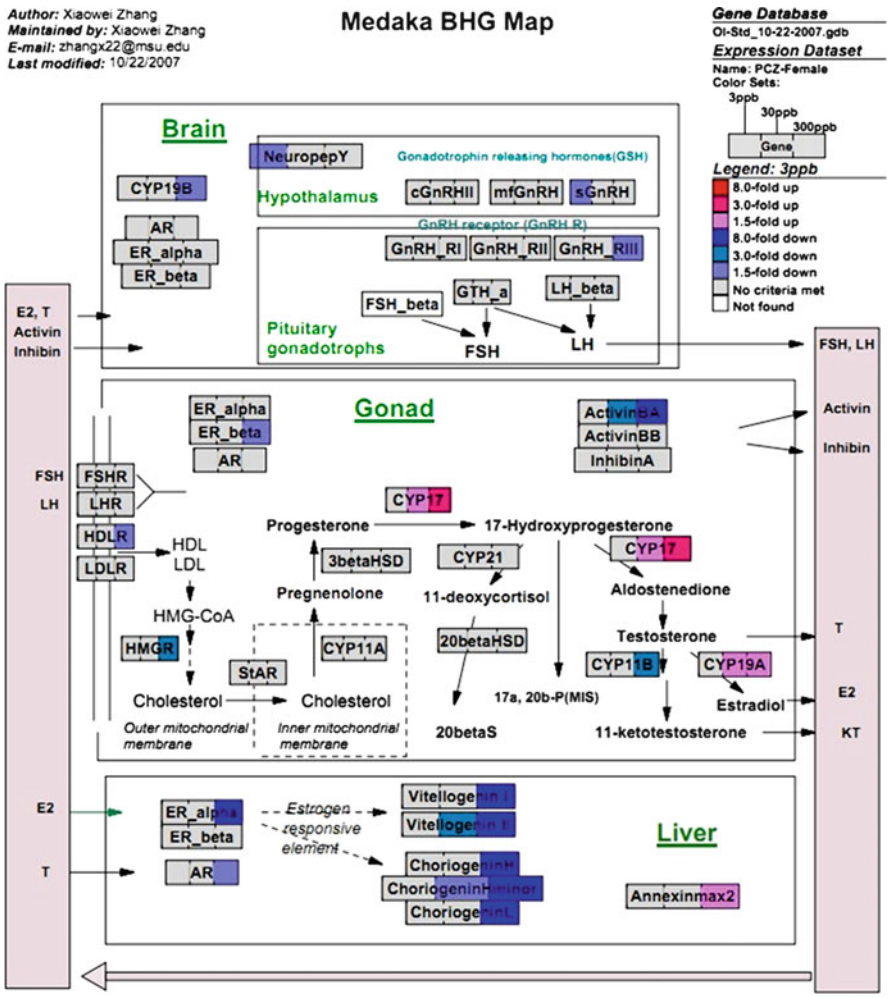


Fig. 4 Medaka hypothalamic–pituitary–gonadal (HPG) axis model and striped view of concentration-dependent response profiles in prochloraz (PCZ) exposed female Japanese medaka (adapted from [56]). Gene expression data from medaka treated with 3.0, 30, and 300 μg PCZ/L are shown as striped color sets on the selected endocrine pathways along the medaka HPG axis. The legend listed in the *upper right* corner of the graph describes the order of the three PCZ concentrations and the eight colors designating different fold thresholds. Abbreviations: *LH* luteinizing hormone, *FSH* follicle-stimulating hormone, *E2* 17β-estradiol, *T* testosterone, *HDL* high-density lipoproteins, *LDL* low-density lipoproteins

fecundity by TRB and FAD were differentiated by a time-course exposure study aided with the Japanese medaka HPG-PCR array [57]. Both TRB and FAD caused lesser mRNA expression of vitellogenin and choriogenin (CHG) in the liver of females. Exposure to FAD for 8 h resulted in an eightfold and 71-fold down-regulation of expression of estrogen receptor α (ERα) and choriogenin L (CHG L),

respectively, in female liver. The downregulation of estrogen-related genes was not observed until 32 h of TRB exposure. These results support the hypothesis that FAD reduces plasma E2 more quickly by inhibiting aromatase enzyme activity than does TRB, which inhibits the production of the E2 precursor testosterone [57].

In Situ Hybridization

In addition to the Japanese medaka real-time PCR array system, a whole-animal tissue section *in situ* hybridization (ISH) system using radiolabeled probes was developed to detect differential gene expression among tissues of Japanese medaka by Tompsett *et al*. [58]. The ISH method not only allows the measurement of tissue-specific gene expression in a whole-animal model but also provides cellular morphological information in the same organism. Furthermore, a fluorescence *in situ* hybridization (FISH) methodology using fluorescence-labeled riboprobes was developed by Park *et al*. [59] to evaluate gene expression profiles simultaneously in multiple target tissues of Japanese medaka. In this optimized FISH method, confocal fluorescence microscopy was optimized to reduce the autofluorescence signal. Using the aromatase inhibitor fadrozole as a model chemical and gonadal aromatase (CYP19a) as a model gene, the optimized FISH method revealed tissue-specific expression of the CYP19a gene and differentiated the abundance of CYP19a mRNA among cell types. Expression of CYP19a was found to be primarily associated with early stage oocytes, and expression gradually decreased with increasing maturation. In females exposed to 500 ng 17α-ethinylestradiol (EE2)/L, the downregulation of CYP19a expression in ovary was found to be a result of tissue degeneration, specifically a decrease in the number of cells (previtellogenic oocytes) where CYP19a mRNAs are primarily transcribed, rather than to a decrease in expression per cell [60]. These examples suggested that FISH combined with histology enables elucidation of molecular effects of chemicals by associating changes in gene expression with histological effects at tissues and/or cellular level.

Hepatic Transcript Index

The large dimensional dataset of transcriptional profiles generated from toxicogenomics studies can not only be useful in revealing toxic mechanisms or describing pollutant-specific molecular fingerprints but can also be utilized to assess the risk of pollution. To develop the environmental threshold of toxicants, conventional ecological risk assessment theories usually employ toxicity endpoints of mortality, growth, and reproduction, which are directly relevant to the ecological outcomes. To reduce the dimension of gene expression data, a new concept of hepatic transcript index (HTI) was developed to facilitate the application of toxicogenomic data in risk assessment. After investigating a group of chemical-induced effects at

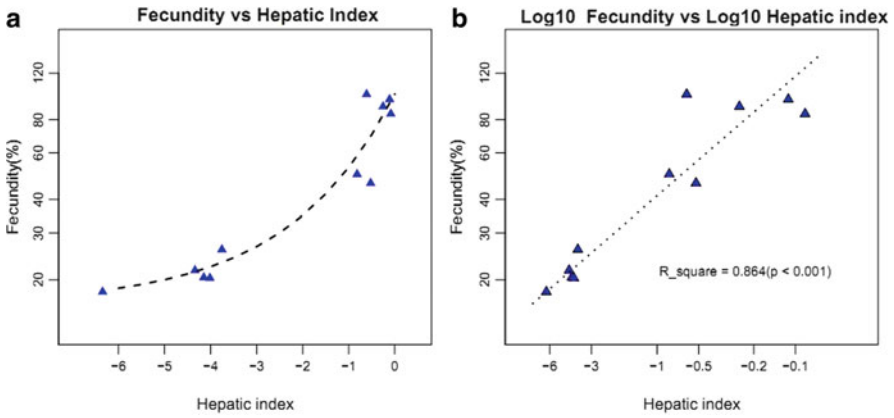


Fig. 5 Relationship between fecundity and gene expression in livers of female Japanese medaka (adapted from [56]). (**a**) Fecundity *vs*. hepatic transcript index (HTI), the broken line shows the trend of data. (**b**) Simple linear regression of $\log_{10}$-transformed fecundity and HTI. The functions describing the relationship are hepatic index = 0.236 *$\log_{10}$ (ERα) + 0.326 *$\log_{10}$ (VTG I) + 0.537 *$\log_{10}$ (VTG II) + 0.472 *$\log_{10}$ (CHG L) + 0.343 *$\log_{10}$ (CHG H) + 0.457 *$\log_{10}$ (CHG HM). The formula for the regression model was $\log_{10}$ (fecundity) = 1.616−0.4493 * $\log_{10}$(−HTI)

the Japanese medaka HPG axis, which included ketoconazole, prochloraz, EE2, TRB, and FAD, HTI in females was found to display a significant linear relationship with fish fecundity [56]. In this analysis, six hepatic genes were observed to be closely correlated at the mRNA expression level across different treatment, which included ERα, VTG I, VTG II, CHG L, CHG H, and CHG HM. Principal component analysis on the mRNA expression of the selected hepatic genes among chemical treatments revealed that the first principle component (PC1) explained 96.3% of the variance among the six genes. The HTI is a sum of log-transformed expression levels of the six hepatic genes weighted by the PC1 factor, which represents the overall expression level of this cluster of gene (Fig. 5). The significant linear relationship between log-HTI and log-fecundity ($r^2 = 0.864$) suggested that the HTI within the HPG axis could be a good indicator of adverse effects at ecological fitness and has potential to be incorporated into ecological risk assessment and regulatory framework.

Application

Cell-based assays and small fish models are useful tools to assess the toxicity and risk(s) of large numbers of pollutants in aquatic systems, such as environmental pharmaceuticals. Pharmaceuticals in the aquatic environment have gained increasing public concern for their potential consequences on human and ecosystem health. These chemicals, which have the potential to alter the endocrine system in

humans or wildlife, could eventually lead to changes in reproductive fitness. Using the H295R cell line and the small fish model medaka, it has been shown that certain pharmaceuticals used in Korea could affect the steroidogenic pathway and alter sex hormone balance although the current concentrations of these pharmaceuticals that occur in Korean rivers are much less than the thresholds for effects on the endpoints [61, 62].

Acknowledgments The research was supported by a New-Faculty Start-up grant from Nanjing University, China, a Discovery Grant from the National Science and Engineering Research Council of Canada (Project #326415-07) and a grant from the Western Economic Diversification Canada (Project #6578 and 6807). The authors wish to acknowledge the support of an instrumentation grant from the Canada Foundation for Infrastructure. Prof. Giesy was supported by the Canada Research Chair program and a large Chair Professorship at the Department of Biology and Chemistry and State Key Laboratory in Marine Pollution, City University of Hong Kong.

References

1. Matthiessen P, Babut M, Batley G, Douglas M, Fawell J, Hommen U, Hutchinson TH, Janssen M, Maycock D, Reiley M, Schneider U, Weltje L (2009) Water and sediment EQS derivation and application. In: Crane M, Matthiessen P, Maycock DS, Merrington G, Whitehouse P (eds) Derivation and use of environmental quality and human health standards for chemical substances in water and soil, environmental standards, Workshop proceedings. CRC, Boca Raton, FL, pp 47–103
2. Kwok KW, Leung KM, Lui GS, Chu SV, Lam PK, Morritt D, Maltby L, Brock TC, Van den Brink PJ, Warne MS, Crane M (2007) Comparison of tropical and temperate freshwater animal species' acute sensitivities to chemicals: Implications for deriving safe extrapolation factors. Integr Environ Assess Manag 3:49–67
3. Ellersieck MR, Asfaw A, Mayer FL, Krause GF, Sun K, Lee G (2003) Acute-to-chronic estimation (ACE v2.0) with time-concentration-effect models: user manual and software. EPA/600/R-03/107. U.S. Environmental Protection Agency, National Health and Environmental Effects Research Laboratory, Gulf Ecology Division, Gulf Breeze, FL
4. Giesy JP, Graney RL (1989) Recent developments in and intercomparisons of acute and chronic bioassays. Hydrobiologia 188(189):21–60
5. Giesy JP, Solomon KR, Coates JR, Dixon KR, Giddings JM, Kenaga EE (1999) Chlorpyrifos: ecological risk assessment in North American aquatic environments. Rev Environ Contam Toxicol 160:1–129
6. Warne St Jr (2001) Derivation of the Australian and New Zealand water quality guidelines for toxicants. Australas J Ecotoxicol 7:123–136
7. Holdway DA (1996) The role of biomarkers in risk assessment. Hum Ecol Risk Assess 2:263–267
8. McCarty LS, Munkittrick KR (1996) Environmental biomarkers in aquatic toxicology: fiction, fantasy, or functional? Hum Ecol Risk Assess 2:268–274
9. Kenaga EE (1982) Predictability of chronic toxicity from acute toxicity of chemicals in fish and aquatic invertebrates. Environ Toxicol Chem 1:347–358
10. Call DJ, Brooke LT, Knuth ML, Poirier SH, Hoglund MD (1985) Fish sub-chronic toxicity prediction model for industrial organic chemicals that produce narcosis. Environ Toxicol Chem 4:335–341
11. McCarty LS (1986) The relationship between aquatic toxicity QSARs and bioconcentration for some organic chemicals. Environ Toxicol Chem 5:1071–1080

12. Abernethy SG, Mackay D, MaCarty LCS (1988) "Volume fraction" correlation for narcosis in aquatic organisms: the key role of partitioning. Environ Toxicol Chem 7:469–481
13. Solomon KR, Giesy JP, Jones PD (2000) Probabilistic risk assessment of agrochemicals in the environment. Crop Prot 19:649–655
14. Wheeler JR, Grist EPM, Leung KMY, Morritt D, Crane M (2002) Species sensitivity distribution: data and model choice. Mar Pollut Bull 45:192–202
15. Mackay D, Shiu WY, Ma KC (1992) Illustrated handbook of physico-chemical properties and environmental fate for organic chemicals, vol 1, Monoaromatic hydrocarbons, chlorobenzenes and PCBs. Lewis Publishers, Chelsea, MI
16. Safe S (1990) Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). Crit Rev Toxicol 21:51–88
17. Safe S (1984) Polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs): biochemistry, toxicology and mechanism of action. Crit Rev Toxicol 13:319–395
18. US-EPA (1981) Interim evaluation of health risks associated with emissions of tetrachlorinated dioxins from municipal waste resource recovery facilities. U.S. Environmental Protection Agency, Office of the Administrator, Washington DC
19. US-EPA (1982) PCB disposal by thermal destruction. U.S. Environmental Protection Agency, National Technical Information Service, Springfield, VA, PB 82-241860
20. US-EPA (2003) Framework for the application of toxicity equivalence methodology for polychlorinated dioxins, furans and biphenyls in ecological risk assessment (external review draft). U.S. Environmental Protection Agency, Washington DC. Online available at http://cfpub.epa.gov/ncea/raf/recordisplay.cfm?deid=55669
21. Olie K, Van den Berg M, Hutzinger O (1983) Formation rate of PCDD and PCDF from combustion process. Chemosphere 12:627–636
22. Ahlborg UG (1989) Nordic risk assessment of PCDDs and PCDFs. Chemosphere 19:603–608
23. Kutz W, Barnes DG, Bretthauer EW, Bottimore DP, Greim H (1990) The international toxic equivalency factor (I-TEF) method for estimating risks associated with exposures to complex mixtures of dioxins and related compounds. Toxicol Environ Chem 26:99–109
24. Barnes DG (1991) Toxic equivalents and EPA's risk assessment of 2,3,7,8-TCDD. Sci Total Environ 104:73–86
25. US-EPA (2003) Toxicity reference values (TRVs) for mammals and birds based on selected aroclors. U.S. Environmental Protection Agency, Region 5, Chicago, IL
26. Van den Berg M, Birnbaum L, Bosveld ATC, Brunström B, Cook P, Feeley M, Giesy JP, Hanberg A, Hasegawa R, Kennedy SW, Kubiak T, Larsen JC, van Leeuwen FX, Liem AK, Nolt C, Peterson RE, Poellinger L, Safe S, Schrenk D, Tillitt D, Tysklind M, Younes M, Waern F, Zacharewski T (1998) Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. Environ Health Perspect 106:775–792
27. Van Leeuwen FXR (1997) Derivation of toxic equivalency factors (TEFs) for dioxin-like compounds in humans and wildlife. Organohalogen Compd 34:237–240
28. Dix DJ, Houck KA, Martin MT, Richard AM, Setzer RW, Kavlock RJ (2007) The ToxCast program for prioritizing toxicity testing of environmental chemicals. Toxicol Sci 95:5–12
29. Sanderson JT, Seinen W, Giesy JP, van den Berg M (2000) 2-chloro-*S*-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: a novel mechanism for estrogenicity? Toxicol Sci 54:121–127
30. Hilscherova K, Jones PD, Gracia T, Newsted JL, Zhang XW, Sanderson JT, Yu RMK, Wu RSS, Giesy JP (2004) Assessment of the effects of chemicals on the expression of ten steroidogenic genes in the H295R cell line using real-time PCR. Toxicol Sci 81:78–89
31. Zhang X, Yu RM, Jones PD, Lam GK, Newsted JL, Gracia T, Hecker M, Hilscherova K, Sanderson T, Wu RS, Giesy JP (2005) Quantitative RT-PCR methods for evaluating toxicant-induced effects on steroidogenesis using the H295R cell line. Environ Sci Technol 39:2777–2785

32. Gracia T, Hilscherova K, Jones PD, Newsted JL, Zhang X, Hecker M, Higley EB, Sanderson JT, Yu RMK, Wu RSS, Giesy JP (2006) The H295R system for evaluation of endocrine-disrupting effects. Ecotoxicol Environ Saf 65:293–305
33. Hecker M, Newsted JL, Murphy MB, Higley EB, Jones PD, Wu R, Giesy JP (2006) Human adrenocarcinoma (H295R) cells for rapid *in vitro* determination of effects on steroidogenesis: hormone production. Toxicol Appl Pharmacol 217:114–124
34. Sanderson JT, Aarts JMMJ, Brouwer A, Froese KL, Denison MS, Giesy JP (1996) Comparison of Ah receptor-mediated luciferase and ethoxyresorufin-*O*-deethylase induction in H4IIE cells: implications for their use as bioanalytical tools for the detection of polyhalogenated aromatic hydrocarbons. Toxicol Appl Pharmacol 137:316–325
35. Ferrero M, Castano A, Gonzalez A, Sanz F, Becerril C (1998) Characterization of RTG-2 fish cell line by random amplified polymorphic DNA. Ecotoxicol Environ Saf 40:56–64
36. Sanchez P, Llorente MT, Castano A (2000) Flow cytometric detection of micronuclei and cell cycle alterations in fish-derived cells after exposure to three model genotoxic agents: mitomycin C, vincristine sulfate and benzo[*a*]pyrene. Mutat Res 465:113–122
37. Veldhoen N, Skirrow RC, Osachoff H, Wigmore H, Clapson DJ, Gunderson MP, Van AG, Helbing CC (2006) The bactericidal agent triclosan modulates thyroid hormone-associated gene expression and disrupts postembryonic anuran development. Aquat Toxicol 80:217–227
38. Zhou B, Liu W, Wu RS, Lam PK (2005) Cultured gill epithelial cells from tilapia (*Oreochromis niloticus*): a new *in vitro* assay for toxicants. Aquat Toxicol 71:61–72
39. Yan CH, Chan KM (2002) Characterization of zebrafish metallothionein gene promoter in a zebrafish caudal fin cell-line, SJD.1. Mar Environ Res 54:335–339
40. Tillitt DE, Ankley GT, Verbrugge DA, Giesy JP, Ludwig JP, Kubiak TJ (1991) H4IIE rat hepatoma cell bioassay-derived 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents in colonial fish-eating waterbird eggs from the Great Lakes. Arch Environ Contam Toxicol 21:91–101
41. Peters AK, van Londen K, Bergman A, Bohonowych J, Denison MS, van den Berg M, Sanderson JT (2004) Effects of polybrominated diphenyl ethers on basal and TCDD-induced ethoxyresorufin activity and cytochrome P450-1A1 expression in MCF-7, HepG2, and H4IIE cells. Toxicol Sci 82:488–496
42. Brack W (2003) Effect-directed analysis: a promising tool for the identification of organic toxicants in complex mixtures? Anal Bioanal Chem 377:397–407
43. Schuetzle D, Lewtas J (1986) Bioassay-directed chemical analysis in environmental research. Anal Chem 58:1060A–1075A
44. Hecker M, Hollert H (2009) Effect-directed analysis (EDA) in aquatic ecotoxicology: state of the art and future challenges. Environ Sci Pollut Res Int 16:607–613
45. Freyberger A, Schmuck G (2005) Screening for estrogenicity and anti-estrogenicity: a critical evaluation of an MVLN cell-based transactivation assay. Toxicol Lett 155:1–13
46. Gracia T, Jones PD, Higley EB, Hilscherova K, Newsted JL, Murphy MB, Chan AK, Zhang X, Hecker M, Lam PK, Wu RS, Giesy JP (2008) Modulation of steroidogenesis by coastal waters and sewage effluents of Hong Kong, China, using the H295R assay. Environ Sci Pollut Res Int 15:332–343
47. Higley EB, Newsted JL, Zhang X, Giesy JP, Hecker M (2010) Assessment of chemical effects on aromatase activity using the H295R cell line. Environ Sci Pollut Res Int 17:1137–1148
48. EDSTAC (2011) Endocrine disruptor screening and testing advisory committee final report. Online available at http://www.epa.gov/endo/pubs/edspoverview/finalrpt.htm
49. Zhang X, Newsted JL, Hecker M, Higley EB, Jones PD, Giesy JP (2009) Classification of chemicals based on concentration-dependent toxicological data using ToxClust. Environ Sci Technol 43:3926–3932
50. Simmons SO (2009) Hypoxia response: a model toxicity pathway for high-throughput screening. Toxicol Sci 112:1–3
51. Wittbrodt J, Shima A, Schartl M (2002) Medaka—a model organism from the far East. Nat Rev Genet 3:53–64
52. Ankley GT, Daston GP, Degitz SJ, Denslow ND, Hoke RA, Kennedy SW, Miracle AL, Perkins EJ, Snape J, Tillitt DE, Tyler CR, Versteeg D (2006) Toxicogenomics in regulatory ecotoxicology. Environ Sci Technol 40:4055–4065

53. Villeneuve DL, Larkin P, Knoebl I, Miracle AL, Kahl MD, Jensen KM, Makynen EA, Durhan EJ, Carter BJ, Denslow ND, Ankley GT (2007) A graphical systems model to facilitate hypothesis-driven ecotoxicogenomics research on the teleost brain-pituitary-gonadal axis. Environ Sci Technol 41:321–330
54. Zhang X, Hecker M, Park JW, Tompsett AR, Newsted J, Nakayama K, Jones PD, Au D, Kong R, Wu RS, Giesy JP (2008) Real-time PCR array to study effects of chemicals on the hypothalamic-pituitary-gonadal axis of the Japanese medaka. Aquat Toxicol 88:173–182
55. Ankley GT, Johnson RD (2005) Small fish models for identifying and assessing the effects of endocrine-disrupting chemicals. ILAR J 45:469–483
56. Zhang X, Hecker M, Tompsett AR, Park JW, Jones PD, Newsted J, Au D, Kong R, Wu RS, Giesy JP (2008) Responses of the medaka HPG axis PCR array and reproduction to prochloraz and ketoconazole. Environ Sci Technol 42:6762–6769
57. Zhang X, Hecker M, Park JW, Tompsett AR, Jones PD, Newsted J, Au DW, Kong R, Wu RS, Giesy JP (2008) Time-dependent transcriptional profiles of genes of the hypothalamic-pituitary-gonadal axis in medaka (*Oryzias latipes*) exposed to fadrozole and 17β-trenbolone. Environ Toxicol Chem 27:2504–2511
58. Tompsett AR, Park JW, Zhang X, Jones PD, Newsted JL, Au DW, Chen EX, Yu R, Wu RS, Kong RY, Giesy JP, Hecker M (2009) *In situ* hybridization to detect spatial gene expression in medaka. Ecotoxicol Environ Saf 72:1257–1264
59. Park JW, Tompsett A, Zhang X, Newsted JL, Jones PD, Au D, Kong R, Wu RS, Giesy JP, Hecker M (2008) Fluorescence *in situ* hybridization techniques (FISH) to detect changes in CYP19a gene expression of Japanese medaka (*Oryzias latipes*). Toxicol Appl Pharmacol 232:226–235
60. Park JW, Tompsett AR, Zhang X, Newsted JL, Jones PD, Au DW, Kong R, Wu RS, Giesy JP, Hecker M (2009) Advanced fluorescence *in situ* hybridization to localize and quantify gene expression in Japanese medaka (*Oryzias latipes*) exposed to endocrine-disrupting compounds. Environ Toxicol Chem 28:1951–1962
61. Han S, Choi K, Kim J, Ji K, Kim S, Ahn B, Yun J, Choi K, Khim JS, Zhang X, Giesy JP (2010) Endocrine disruption and consequences of chronic exposure to ibuprofen in Japanese medaka (*Oryzias latipes*) and freshwater cladocerans *Daphnia magna* and *Moina macrocopa*. Aquat Toxicol 98:256–264
62. Ji K, Choi K, Lee S, Park S, Khim JS, Jo EH, Choi K, Zhang X, Giesy JP (2010) Effects of sulfathiazole, oxytetracycline and chlortetracycline on steroidogenesis in the human adreno-carcinoma (H295R) cell line and freshwater fish *Oryzias latipes*. J Hazard Mater 182:494–502
63. Zhang X, Wu RS, Fu W, Xu L, Lam PK (2004) Production of reactive oxygen species and 8-hydroxy-2′deoxyguanosine in KB cells co-exposed to benzoapyrene and UV-A radiation. Chemosphere 55:1303–1308

Perfluorinated Compounds

Christopher Lau

Abstract Perfluorinated compounds such as the perfluoroalkyl acids (PFAAs) and their derivatives are important man-made chemicals that have wide consumer and industrial applications. They are relatively contemporary chemicals, being in use only since the 1950s and until recently have been considered as biologically inactive. However, during the past decade, their global distribution, environmental persistence, presence in humans and wildlife, and adverse health effects in laboratory animals have come to light, generating scientific, regulatory, and public interest on an international scale. This chapter will provide a brief overview of recent advances in understanding environmental and human exposure, toxicology, and modes of action for this class of compounds in animal models, as well as a summary of epidemiological findings to date.

Keywords Perfluorinated compounds · Perfluoroalkyl acids · Perfluoroalkyl sulfonates · Perfluorooctane sulfonate · Perfluoroalkyl carboxylates · Perfluorooctanoic acid · Perfluoroalkyl phosphonates

Introduction

Perfluorinated compounds are organic chemicals in which all hydrogens of the carbon chain are substituted by fluorine atoms. Generally, there are two types of perfluorinated compounds, the perfluoroalkanes that are used primarily for

C. Lau
Toxicity Assessment Division, National Health and Environment Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711, USA
e-mail: lau.christopher@epa.gov

A. Luch (ed.), *Molecular, Clinical and Environmental Toxicology*, Experientia Supplementum 101, DOI 10.1007/978-3-7643-8340-4_3,

oxygenation and respiratory ventilation clinically and the perfluoroalkyl acids (PFAAs) that are the subject of this chapter. Environmentally relevant PFAAs are a family of about 30 chemicals that consist of a carbon backbone typically 4–14 atoms in length and a charged functional group composed of either sulfonates, carboxylates, or phosphonates (and to a lesser extent, phosphinates). While many (>100) derivatives of PFAAs (such as alcohols, amides, esters, and acids) are used for industrial and consumer applications, they can be degraded or metabolized to PFAAs as end-stage products. Thus, PFAAs, rather than their intermediates or derivatives, have drawn the most public attention and research interest. The most widely known PFAAs are the eight-carbon (C8) sulfonate (perfluorooctane sulfonate, PFOS) and carboxylate (perfluorooctanoic acid, PFOA), although the C4 and C6 sulfonates, as well as the C4, C6, and C9 carboxylates, have also been used in commerce. The perfluoroalkyl phosphonates (PFPAs) are fairly new for this class of chemicals. They are typically used as leveling and wetting agents, and defoaming additives in the production of pesticides. They were considered biologically inert by the US Environmental Protection Agency until 2006. Mabury and coworkers [1] were the first to report the detection of PFPAs in the environment, and to date, only one additional paper has been published to describe the pharmacokinetics of PFPAs in the rat [2]. Discussion in this chapter will therefore focus on perfluoroalkyl sulfonates (PFSAs) and carboxylates (PFCAs), for which information is readily available. Indeed, in the past few years, an increasing number of reports concerning PFAAs have appeared in the literature, and over a dozen salient topical reviews have been published to highlight the biomonitoring, toxicological, and epidemiological findings for these compounds [3–15]. Hence, this chapter will provide a brief, overarching description of these perfluorinated chemicals, and readers are encouraged to consult the particular review papers for specific details.

Background

Naturally occurring fluorinated organic chemicals are rare. PFAAs are fairly contemporary chemicals, synthesized since the 1950s by electrochemical fluorination of an organic feedstock or by telomerization of tetrafluoroethylene units. Neither of these manufacturing processes is precise, thus yielding a family of target compounds as well as unintended by-products of various carbon-chain lengths and isomers [16]. The unique hydrophobic and oleophobic nature of PFAAs makes these chemicals ideal surfactants [17]. There are over 200 known industrial and consumer applications of PFAAs, including water, soil and oil repellents, lubricants, fire-fighting foams, and emulsifiers used in the production of fluoropolymers. PFAAs were initially considered metabolically inert. They are stable, nonreactive, and do not undergo metabolism. Structurally, they resemble fatty acids. In fact, in the early literature, they were often referred to as perfluorinated fatty acids. They bind to hepatic fatty acid-binding proteins, competing for

binding with the natural ligands [18–20]; however, PFAAs are not known to participate in biochemical reactions that use fatty acids as substrate. They also bind to other proteins in serum, liver, kidney, and testes [21–26]. PFAAs are known to serve as substrates and regulators of renal and hepatic organic anion transporters [27, 28] and as activators of nuclear receptors that regulate fatty acid and glucose metabolism and transport [29–38]. They have also been shown to alter cell membrane fluidity and membrane function via their surfactant effects [39–47], to interfere with intercellular communication through inhibition of gap junctions [48–50], and to disrupt mitochondrial bioenergetics and biogenesis [51–54, 203].

Historically, production of PFAAs is dominated by the C8 chemical, PFOS, and to a lesser extent, PFOA. In 2002, the major manufacturer of PFOS in the USA phased out production of this chemical, leading to a precipitous drop in global production. However, this market void has since been replenished to some extent by Asian (e.g., China) and European producers in recent years. In addition, increased production of PFOA has made it the most common PFAA in commerce. In 2006, the US Environmental Protection Agency initiated the PFOA Stewardship Program with industry, with the goal of eliminating emissions and product content of these chemicals by 2015. To accomplish this goal, shorter carbon-chain PFAAs such as perfluorobutane sulfonate (PFBS) and perfluorohexanoic acid (PFHxA), as well as different chemistries (such as ammonium 4,8-dioxa-3*H*-perfluorononanoate, ADONA [55]), are poised to replace the C8 compounds in commerce.

Environmental Fate and Transport of PFAAs

A summary of global production, emission, and environmental inventory for PFOS was provided by Paul *et al*. [56]. PFCAs are primarily derived from degradation of fluorotelomer alcohols and polyfluoroalkyl phosphates in the atmosphere, soil, and wastewater treatment plant (WWTP) sludge and from landfills [57–67]. The metabolic pathways for some fluorotelomer alcohols in *in vitro* and *in vivo* systems have been summarized [68–70]. These chemicals can be transferred from water to soil and taken up by plants [71, 72]. Armitage *et al*. [73] have recently described a model of global fate and transport of PFCAs. In general, two routes have been proposed to account for the global distribution of PFAAs, including remote regions such as the Arctic. The first hypothesis suggests an indirect atmospheric transport of PFAA precursors and subsequent degradation to PFSAs and PFCAs [74–77], whereas a second hypothesis favors a direct release of PFAAs and long-range ocean water transport [78–81] to the remote locations. While these two hypotheses remain a subject of debate, it is likely that both routes are involved in the distribution of these contaminants. At a local level, Davis *et al*. [82] have constructed a model of PFOA migration from a point source, where PFOA vapor and particulates

are emitted in the air, transported by wind, deposited on the surface soil, and leached to surface water and then to groundwater within the aquifer.

Environmental Exposure of PFAAs

Several reviews have previously summarized the biomonitoring studies on PFAAs in the environment, in wildlife, and in humans [3, 7, 9, 11, 12]. This chapter will only highlight the key features of these descriptions and provide an update of findings since the publication of these reviews. PFAAs are globally distributed and ubiquitously detected in all environmental media, including air, surface and drinking water, soil, sediment, and sludge recovered from wastewater treatment plants (WWTP). A number of Asian, European, and North American studies have documented PFAA particulates and telomer alcohol precursors in indoor air (~450 ng/m^3), house dust (~10–40 μg/g), and ambient air (~800 pg/m^3) [83–90]. Similarly, PFAAs in environmental and tap water have been detected worldwide, and a summary of these findings is available in recent reviews [91, 92]. Typically, PFAAs found in lakes and rivers may range from 0.3 to 2,600 ng/L and in drinking water from 0.1 to 70 ng/L [93–97] (although a PFOA level as high as 3,550 ng/L has been reported in West Virginia [98]). In that regard, health and safety guidelines for PFOS and PFOA in drinking water have been issued recently by various regulatory agencies [99–103]. Recent discoveries of PFAA-contaminated biosoils applied in farms and fields in Germany [104, 105] and in the USA [66, 67, 106] have raised significant research interests and public concerns [107]. These biosoils are derived from sewage sludge generated from municipal and industrial WWTP. Various studies have documented detection of PFAAs in both inflow and outflow of these treatment plants, suggesting that WWTP can be significant sources of these chemicals in the environment [108–112].

Since the seminal findings reported by Giesy and Kannan in 2001 [113–115], numerous studies have documented the widespread contamination of PFAAs in wildlife from the North Pole to the South Pacific. Several recent reviews have summarized these monitoring findings [7, 9] and described the various trends of bioaccumulation [116–120]. Human exposure to PFAAs was initially reported by occupational biomonitoring conducted by the manufacturers [121–123], followed by detection in selected samples from Red Cross blood donors [124]. Subsequently, reports from the National Health and Nutrition Examination Survey (NHANES) conducted by the Centers for Disease Control and Prevention (CDC) revealed significant detection of PFOS, PFOA, perfluorohexane sulfonate (PFHxS), and perfluorononanoic acid (PFNA) in the US general population [125–128]. Serum levels of these chemicals from several NHANES reports are summarized in Table 1 and compared to levels in sera from occupational exposures and exposures of residents at PFAA-contaminated areas. Serum PFAA levels are understandably highest in the production workers who are routinely exposed to these chemicals. In the general population, levels of PFOS are higher than those of PFOA, and the

Table 1 Summary of major PFAAs reported in the National Health and Nutrition Examination Surveys (NHANES) and examples of exposure to residents at contaminated areas and occupational exposure [105, 121–123, 125–128, 158, 208]

	PFOS	PFOA	PFHxS	PFNA
NHANES 1999–2000	30.4	5.2	2.1	0.5
NHANES 2001–2002*	20.8	3.7	2.8	0.6
NHANES 2001–2002* (children)	30.5–42.5	6.1–7.6	4.5–18.7	0.6–1.2
NHANES 2003–2004	20.7	4.0	1.9	1.0
NHANES 2005–2006	17.1	3.9	1.7	1.1
NHANES 2007–2008	13.2	4.1	2.0	1.5
Arnsberg, Germany 2006	23.4–30.3	5.1–12.7	1.3–2.7	–
Little Hocking, WV 2007	23	368	–	–
Production workers	1,500–2,000	500–1,000	~500	Unknown

*denote values derived from pooled samples

levels of PFHxS and PFNA are substantially lower. By and large, profiles of PFAA exposure in humans comparable to those seen in the USA have been reported with other populations worldwide [129–136]. Among the four NHANES reports, there is a general trend for decline of serum PFAAs, with the exception of PFNA, the levels of which have doubled in the recent surveys. Such a declining trend is consistent with another report that follows the levels of PFAAs in sera from Red Cross blood donors [137]. Although the data are limited, levels of PFOS and PFOA appear to be higher in children than in adults, suggesting that children may be a vulnerable subpopulation for chemical exposure [126, 136]. Indeed, exposure to PFAAs appears to begin early in life, as PFOS and PFOA in particular have been detected in umbilical cord blood and in breast milk [138–147]. The routes of human exposure to PFAAs remain a subject of debate, although they likely involve migration of chemical from food packaging [148, 149], food intake [150–152], drinking water, and house dust. Exposure models from a recent review [153] suggested that food intake is the major exposure pathway for the general population, while drinking water exposure is dominant for populations near contaminated sites. Tolerable daily intake (TDI) of 100–300 ng/kg (body weight) for PFOS and 0.1–3 μg/kg for PFOA in food has been recommended by the European food regulatory authorities [154–156], and health advisories for PFOS (0.2 μg/L) and PFOA (0.04–0.4 μg/L) in drinking water have been issued by federal and state regulatory agencies in the USA [101–103] and in Europe [157] (Table 2). Considerably higher levels of PFAAs have also been detected among residents in areas, particularly in West Virginia, where contamination in drinking water was found [105, 158], although it is heartening to note that these levels began to decline once mitigation steps were taken.

Pharmacokinetic Disposition of PFAAs

Because of their physicochemical characteristics, most PFAAs possess unique pharmacokinetic properties based on their carbon-chain lengths and functional

Table 2 Recommended tolerable intake (TDI) levels of PFAAs by regulatory bodies

	PFOA	PFOS	References
US Environmental Protection Agency (drinking water)	0.4 μg/L	0.2 μg/L	[103]
Minnesota Department of Health (drinking water)	0.3 μg/L	0.3 μg/L	[102]
New Jersey Department of Environmental Protection (drinking water)	0.04 μg/L	–	[101]
Drinking Water Commission of German Ministry of Health (drinking water)	100 ng/kg BW	100 ng/kg BW	[100]
European Food Safety Authority (food)	1.5 μg/kg BW	150 ng/kg BW	[154]
UK Committee on Toxicity in Food, Consumer Products and the Environment (food)	3 μg/kg BW	300 ng/kg BW	[155, 156]
German Federal Institute for Risk Assessment (food)	100 ng/kg BW	100 ng/kg BW	[157]

BW body weight

groups, as well as the species, gender, and age of the subjects evaluated. Animal studies (typically with rodents) of various PFAAs have shown that they are well absorbed orally (within hours), are not metabolized, undergo extensive enterohepatic circulation, and readily cross the placenta. PFAAs are poorly eliminated (especially the long-chain PFAAs), and elimination is primarily via urinary excretion [159–161]. These chemicals are distributed mainly to the serum, kidney, and liver, with liver concentrations being several times higher than serum concentrations (with the exception of PFBA, perfluorobutanoate). The volume of distribution at steady state suggests that PFAA distribution is likely extracellular. These chemicals also have high binding affinity for a variety of proteins [17–25].

The elimination half-lives of several PFAAs in animal models and humans are summarized in Table 3 [158, 161–175, 179, 203]. In general, the rate of elimination is enhanced with decreasing carbon-chain length. Thus, the elimination half-lives of PFBS, PFBA, and PFHxA are shorter than those of PFOS, PFOA, and PFNA among most species examined. The lone exception is PFHxS, where limited data indicate that it does not follow this trend. Across the species evaluated, the rate of elimination is slowest in humans, with the half-life rank order being humans > monkey > mouse > rat. Few gender differences in PFAA clearance are observed in humans or monkeys. In contrast, marked sex differences are observed in the rat, particularly with PFCAs. Most notably, the half-lives of PFNA and PFOA in female rats are 20 and 50 times shorter than those in males, respectively. Interestingly, the gender difference in PFOA elimination is developmentally regulated in rats. The rapid elimination seen in female rats develops between 3 and 5 weeks of age [167]. Smaller sex differences are generally seen with PFSAs. On the other hand, the sex differences in PFAA elimination are consistently much smaller in the mouse than in the rat. In that regard, the mouse resembles humans more closely and thus provides a rodent model more amenable for extrapolation of results from toxicological studies, particularly those focusing on reproductive and developmental toxicity where pharmacokinetics in the pregnant females play a major role in determining the exposure of the conceptus.

Table 3 Summary of serum/plasma elimination half-lives of various PFAAs

Species	PFBS		PFHxS		PFOS		PFBA		PFHxA		PFOA		PFNA	
	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
Rat	4.0 h	4.5 h	0.8 days		62–71 days	38–41 days	1.0–1.8 h	6–9 h	0.42 h	1.0 h	2–4 h	4–6 days	1.4 days	30.6 days
Mouse			25–27 days	28–30 days	31–38 days	36–43 days	3 h	12 h			17 days	19 days	25.8–68.4 days	34.3–68.9 days
Rabbit											7 h	5.5 h		
Dog											8–13 days	20–30 days		
Monkey	3.5 days	4.0 days	87 days	141 days	110 days	132 days	1.7 days		0.1–0.8 days	0.2–1.5 days	30 days	21 days		
Human	1 month		8.5 years		5.4 years		3 days				2.3–3.8 years			

PFBS perfluorobutane sulfonate; *PFHxS* perfluorohexane sulfonate; *PFOS* perfluorooctane sulfonate; *PFBA* perfluorobutanoate; *PFHxA* perfluorohexanoic acid; *PFOA* perfluorooctanoic acid; *PFNA* perfluorononanoic acid. References for rat [161, 163, 167, 170, 172–175, 179, 203], mouse [170, 171, 175, 179, 203], rabbit [167], dog [160], monkey [164, 170, 172, 173, 179, 203] and humans [158, 168, 169, 171]

The mechanisms underlying the sex difference for PFCA elimination in the rat are presently under active investigation and likely are related to renal clearance of the chemicals [176]. A number of studies have implicated the involvement of organic anion transporters (OATs) that are regulated by sex hormones [161, 177, 178]. A recent study by Weaver *et al*. [27] indicated that OAT1 and OAT3 are involved in renal secretion of perfluoroheptanoic acid (PFHpA), PFOA, and PFNA, while OATP1a1 contributes to the reabsorption of PFOA, PFNA, and perfluorodecanoic acid (PFDA). At present, it is not clear whether the diminished sex difference noted in the mouse for PFCA clearance is also related to these transporters. Ljubojevic *et al*. [178] reported that the renal expression pattern of OAT2 in the mouse resembles that in the rat, and both are under regulation by sex hormones. In contrast, Buist *et al*. [177] indicated that renal OAT2 mRNA levels are markedly higher in female than in male rat, but there is no sex difference in OAT2 expression in the mouse kidney [180]. Additional studies are needed to resolve this issue. On the other hand, it is encouraging that these transporters (such as OAT4 and urate transporter 1, URAT1) have been shown to play a key role in renal reabsorption of PFCA in humans [181], suggesting a potential common mechanism across species.

Toxicological Findings with PFAAs

The toxicology of PFOS and PFOA has been extensively reviewed in the past few years [5, 6, 9, 10]. Readers are encouraged to consult with these reviews for detailed descriptions. This chapter will highlight findings from primarily mammalian models, provide an update of information, and focus on recent discoveries with other PFAAs. Generally speaking, six major adverse effects have been identified with PFAA exposure in laboratory studies: tumor induction, hepatotoxicity, developmental toxicity, immunotoxicity, endocrine disruption, and neurotoxicity.

Tumor Induction

Neither PFOS, PFHxA, PFOA, nor PFDA is known to be mutagenic [5, 182–185]. A recent study suggested the genotoxic potential of PFOA in HepG2 cells but was likely associated with oxidative stress and ROS production [186]. However, DNA damage was observed only at high concentrations of PFOA (50–100 μM). In addition, while intracellular ROS production was increased by PFOA and PFOS in another study [187], no corresponding DNA damage was observed. PFBS and PFHxA did not generate ROS or DNA damage. PFNA caused DNA damage only at a cytotoxic concentration.

Significant positive trends were noted in the incidence of hepatocellular adenoma in rats exposed to high dietary doses of PFOS (20 ppm or 1.5 mg/kg/day) for 2 years [182], although this evidence was considered equivocal for carcinogenicity [156].

Significant increases in mammary fibroadenoma and adenoma were seen in the low-dose groups (0.5 and 2 ppm), but there was no dose–response relationship with this effect, as increases in the 5 ppm dose group were not statistically significant and a slight decrease of tumor incidence was seen at 20 ppm.

A significant increase in the incidence of mammary fibroadenoma in rats exposed to dietary doses of 30 or 300 ppm (16.1 mg/kg/day) PFOA for 2 years was also reported [188], but these findings were subsequently refuted by a review panel [189]. On the other hand, significant increases in the incidence of liver adenomas, pancreatic acinar cell tumors, and testicular (Leydig) cell adenomas were seen in rats exposed chronically to 300 ppm of PFOA in diet [5]. This liver–pancreas–testes triad of tumors is typical of many agonists of the peroxisome proliferator-activated receptor-alpha (PPARα). The hepatocellular tumors are most likely related to activation of the PPARα molecular pathway. Tumors observed in the testis have been associated with elevation of hepatic aromatase activity, leading to increases of serum estradiol, in concert with testicular growth factors [190, 191]. The mechanism(s) responsible for the PFOA-induced pancreatic tumors remain the subject of active investigation. In addition, using a unique tumor model of rainbow trout, Tilton *et al*. [192] showed that chronic PFOA exposure for 30 weeks resulted in enhanced liver tumor incidence, although the dose employed in this study (1,800 ppm or 50 mg/kg/day) was quite high.

Hepatotoxicity

Hepatomegaly primarily involving hepatocytic hypertrophy is perhaps a hallmark PFAA effect in laboratory animals, produced by PFOS, PFHxS, PFBS, PFDA, PFNA, PFOA, PFHpA, PFHxA, and PFBA [5, 9, 182, 185, 188, 193–198] and is likely associated with peroxisome proliferation. Chronic exposure to high doses of PFOA and PFOS led to hepatocellular vacuolation, degeneration and necrosis, accumulation of lipid droplets related to altered lipid metabolism and transport, and tumor induction [182, 188]. PFAAs, particularly the PFCAs (C6–C10), are known to induce hepatic peroxisomal fatty acid β-oxidation in rats and mice [194, 199, 200], leading to reduction of serum triglycerides and cholesterol [5, 201]. The hypolipidemic effect of PFOA is due, in part, to the reduced synthesis of cholesterol and an enhanced oxidation of fatty acids in the liver. However, despite an enhanced β-oxidation of fatty acids, Kudo *et al*. [202] have demonstrated an increase of glycerolipids and triglycerides in liver of rats treated with PFOA, which may be linked to increased *de novo* synthesis [202, 303]. The increase in triglyceride synthesis and accumulation in the liver, but a reduced level in circulation, prompted these investigators to suggest impaired hepatic secretion of triglycerides. In view of recent findings regarding the effects of PFAAs on various transporter proteins, this hypothesis is entirely conceivable, although future research on hepatic transporters that traffic lipids and other macromolecules are needed to clarify this issue. On the

other hand, the potential adverse effects of the apparent "fatty liver" produced by PFOA remain to be determined.

Recent toxicogenomic analyses of rodent livers after exposure to PFOA and PFOS revealed a strong PPARα signature [204–206] and supported previous findings from an *in vitro* system [207]. The involvement of PPARα signaling was further confirmed with studies using a transgenic mouse model where PPARα function was deleted [36, 209–210]. However, in contrast to the responses elicited by the potent PPARα agonist WY14, 643, where 99% of the observed changes in gene expression were eliminated in the PPARα-null mice, about 20% of the PFOA-induced genomic responses were still detected in the PPARα-knock out mice, suggesting a PPARα-independent mechanism for the perfluorinated chemical [211]. Further examination of the PPARα-independent genomic responses implicated another nuclear receptor, the constitutive androstane receptor (CAR) [35, 38], which is known to be involved in xenobiotic metabolism. Potential involvement of other nuclear receptors such as pregnane X receptor (PXR) and liver X receptor (LXR) in PFAA-induced hepatic responses is currently under active investigation [33, 213]. These nuclear receptors (PPAR, CAR, PXR, and LXR) are important regulators of fatty acid transport and metabolism, xenobiotic metabolism, and cholesterol and glucose homeostasis, which can readily account for some of the cellular responses elicited by PFAAs.

Developmental Toxicity

The adverse reproductive and developmental effects derived from exposure to PFAAs have been summarized in detail in previous reviews [6, 9]; thus, only salient features and updates of these effects are described here. Exposure to PFOS or PFOA during pregnancy in rats and mice produced overt anatomical defects in offspring (such as cleft palate) only at high doses, while other morphological abnormalities noted in fetuses chiefly reflected developmental delays [214–217]. Early pregnancy loss was noted with PFOA or PFBA exposure but only at very high doses, and the etiology of this effect is not clear. No frank terata or fetotoxicity was observed after gestational exposure to PFBA or PFDA [218, 219]. In contrast, when dams exposed to PFOS were allowed to give birth, dose-dependent deleterious effects were seen in the newborns [220, 221]. Although all pups were born alive and active, those exposed to high doses (5 or 10 mg/kg) became moribund within the ensuing hours and died soon afterward. Survival improved with lower PFOS exposure, but postnatal growth of surviving pups was somewhat stunted, and reductions of circulating thyroid hormones were observed. The PFOS-induced hypothyroxinemia was confirmed in a recent study that correlated PFOS accumulation with hormonal imbalance [222]. In addition, a critical prenatal window of PFOS exposure toward late gestation was noted for the adverse postnatal effects [223], potentially implicating immaturity of the newborn lung and pulmonary insufficiency as causes for neonatal death. However, no evidence of changes in lung phospholipids or markers

for alveolar differentiation was found to support underdevelopment of the neonatal lung [224]. Alternatively, because PFOS itself is a surfactant, one can speculate that the synthetic chemical may interact with endogenous pulmonary surfactant, thereby interrupting its function to facilitate the inflation of the neonatal lung after birth. The observation of a preferential accumulation of PFOS in the fetal lung adds support to this hypothesis [225]. Importantly, Xie *et al*. [41, 45] reported that PFOS (and to a lesser extent, PFOA) had a strong tendency to interact with dipalmitoylphosphatidylcholine (DPPC) and partition into lipid bilayers. Because DPPC is a major component of pulmonary surfactant, it is possible that such PFOS–DPPC physical interactions may interfere with the physiological function of pulmonary surfactant. However, the evidence available at present is still circumstantial, and definitive results from *in vivo* studies are needed to confirm respiratory distress related to impaired lung surfactant function as a pathophysiological mechanism for the PFOS-induced neonatal mortality.

In contrast to PFOS, the reproductive toxicological findings in rats exposed to PFOA were rather unremarkable [228], which might have been related to the unique ability of female rats to clear the chemical efficiently (half-life of 2–4 h, Table 3). Indeed, in mice, where elimination of PFOA is considerably less rapid (half-life of 17 days, Table 3) and chemical accumulation occurs in the females, a profile of neonatal mortality was noted when pregnant dams were exposed to high doses of PFOA (>10 mg/kg) [216]. The newborn mice appeared to survive slightly better and died less abruptly than those exposed to PFOS, perhaps partly due to a lesser effect of PFOA in interrupting lung surfactant function [41]. Among the surviving mice exposed to lower PFOA doses, neonatal growth deficits and developmental delays were seen. Evaluation of mammary differentiation of the nursing dams indicated significant reductions at postnatal day 10, suggesting that abnormal lactation function may play a role in the growth retardation of their offspring [229]. However, results from a cross-fostering study indicated that the developmental deficits seen in mouse pups were largely due to prenatal exposure to PFOA [230]. Interestingly, although growth impairment was noted in neonates exposed to relatively high doses (3–10 mg/kg) of PFOA during gestation, those exposed to low doses (0.01–0.3 mg/kg) displayed significant increases in body weight and serum insulin and leptin concentrations during mid-life [231]. In contrast, PFOA exposure of adult mice at comparable doses did not produce any weight effect, indicating a specificity of chemical perturbation during developmental periods. These paradoxical findings are intriguing and will require further elaboration but may reflect the subtle alterations of developmental programming of metabolic processes, where the adverse outcomes are manifested latently at adult ages, akin to a theory advanced by Barker [232]. In addition, mammary gland development in female mouse offspring exposed to PFOA was significantly delayed, leading to persistent abnormalities [233]. The functional sequelae of these morphological abnormalities are currently unknown, and future work should explore whether the lactational capability of these female mice (exposed to PFOA prenatally) is negatively impacted.

PFOA is known to be a PPARα agonist. In view of the important roles of this nuclear receptor in reproduction and development [234], Abbott and colleagues investigated the role of the PPARα molecular pathway in PFOA-induced developmental toxicity using a transgenic PPARα-null mouse model [235]. Wild-type (129 S1/SvlmJ) mice were slightly more sensitive to PFOA toxicity than CD-1 mice [216], but both strains displayed similar neonatal mortality, growth deficits, and developmental delays. However, these adverse outcomes were markedly attenuated in the PPARα-null mice, suggesting that PFOA developmental toxicity is dependent on expression of PPARα. In contrast, results from a follow-up study by the same investigators indicated that the developmental toxicity of PFOS was not dependent on this nuclear receptor function [236], thus possibly delineating distinct modes of action between PFCAs and PFSAs regarding their developmental effects. This contention is further supported by a recent developmental study with PFNA, where a near-identical profile of PPARα-dependent responses was detected [237].

Compared to long-chain PFAAs (>C8), the short-chain chemicals are much less toxic to the developing animal, in part due to their faster rate of clearance (Table 3). Thus, even at very high doses of PFBA (350 mg/kg, intended to match the body burden of PFOA), neither neonatal survival nor postnatal growth was compromised, although maternal hepatomegaly was detected (indicating the effectiveness of the PFBA dose regimen) and neonatal liver weight was transiently elevated [218]. A similar lack of overt reproductive and developmental toxicity has been reported for PFHxA [185], PFBS [196], PFHxS [239], and ADONA [55].

Immunotoxicity

DePierre and colleagues were the first to demonstrate the immunotoxic effects of PFOA in the C57BL/6 mouse, where thymic and splenic atrophy associated with an arrest of thymocyte and splenocyte proliferation and a marked reduction of cell populations were observed after subchronic dietary exposure to the chemical [240–242]. These effects appeared to be mediated by PPARα, as the PFOA-elicited alterations of lymphoid organ weight and cellularity were attenuated in the PPARα-null mice [243]. However, the precise role of this nuclear receptor and the extent of its involvement in the immunotoxicity of PFAAs have been challenged recently [244]. Fairley *et al*. [245] reported similar effects of thymic and splenic atrophy and decreased cellularities in BALB/c mice after dermal exposure to PFOA, along with an enhanced hypersensitive IgE response to ovalbumin. These results suggested that exposure to PFOA, although not allergenic itself, might enhance an individual's response to commonly encountered environmental allergens. Son *et al*. [246] administered PFOA in drinking water to ICR mice (50–250 ppm) and demonstrated an immunomodulatory effect of the chemical that altered T-lymphocyte phenotype in the spleen and thymus and elevated gene expression of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6). Similar PFOA-induced immunomodulation was also observed

in C57BL/6J and C57BL/6N female mice, where IgM antibody synthesis was suppressed and IgG titer was elevated in response to a sheep red blood cell (SRBC) challenge [247]. While thymic and splenic atrophy and decreased IgM production were consistently seen in CD-1 (ICR) male mice given PFOA by oral gavage, no effect on production of anti-SRBC antibodies was noted in the rat. Moreover, an increase of serum corticosterone, increases in numbers of peripheral blood neutrophils and monocytes, and a decrease in absolute lymphocyte numbers were found in the PFOA-treated mice. This prompted the investigators to surmise that the immunotoxic responses were secondary to systemic toxicity of the perfluorinated chemical and the attendant stress evoked [248]. However, results from further investigation of this possibility with adrenalectomized mice (thereby removing the element of stress response) indicated that suppression of humoral immunity by PFOA was independent of the elevated serum corticosterone and not likely associated with stress [249].

Adverse immunological outcomes from exposure to PFOA are extended to other PFCAs such as PFNA [250–252]. Subchronic exposure to PFNA in mice led to reduction of lymphoid organ weight, cell cycle arrest, and apoptosis in spleen and thymus, accompanied by impaired production of IL-4 and interferon-γ by splenic lymphocytes and upregulation of IL-1β. Similar PFNA-induced histopathological changes were seen in the rat, along with alterations of serum cytokines, which in turn activated the mitogen-activated protein kinase (MAPK) signaling pathways that modulate the immune system. In addition, the splenic apoptosis caused by PFNA might be associated with oxidative stress, as the level of hydrogen peroxide was increased and superoxide dismutase activity and Bcl-2 protein levels were dramatically decreased in the spleen.

Immunotoxic responses are also detected in rodents treated with PFSA. Suppression of humoral immunity after exposure to a perfluorinated insecticide that can be metabolized to PFOS was reported [253]. Similar immunotoxic findings were extended to mice directly exposed to high doses of PFOS in a diet that produced a serum level of 340 μg/ml, although the effects were less pronounced than those seen with PFOA [254]. Significant immunomodulatory effects of PFOS were also seen in rats, although changes were generally less robust than those seen in mice [255]. Results from low-dose PFOS studies in mice were less definitive. Peden-Adams *et al.* [256] reported that exposure to PFOS by oral gavage in B6C3F1 mice, which produced serum concentrations of 0.09–0.67 μg/ml, suppressed T-cell-dependent (to SRBC challenge) or T-cell-independent (to trinitrophenyl conjugated lipopolysaccharide challenge, TNP-LPS) IgM antibody responses. Similarly, He and colleagues [257–259] showed that PFOS reduced subpopulations of lymphocytes in lymphoid organs and decreased natural killer cell activity in C57BL/6 mice, at exposure that yielded higher serum concentrations of 0.67–121 μg/ml. However, in a more recent study with B6C3F1 mice where PFOS was given in a diet that produced serum concentrations of 0.048 μg/ml, no adverse effects on adaptive immunity were evident [260]. The investigators speculated that routes of chemical administration might have played a role in these apparently disparate findings. Additional work is needed to clarify the low-dose effects of PFAAs on immune functions.

Interestingly, in a preliminary study where PFOS was given to mice at a dose that produced plasma levels of 0.19–0.67 μg/ml, thymus and spleen weights were not altered but the responses of these animals (emaciation and mortality) to influenza A virus challenge were increased significantly, suggesting that host resistance to pathogens was compromised by exposure to the perfluorinated chemical [261]. Detailed immunological mechanisms responsible for this observation remain to be explored. Gestational exposure to PFOS in mice has also been shown to suppress immune function later in life, indicating that the developing immune system is sensitive to PFAA insult and that these functional deficits might not be apparent until the animals reach adulthood [262].

In addition to their effects on adaptive immunity, influences of PFAAs on innate immunity have also been characterized [263, 264]. Short-term treatment with PFOS or PFOA led to significant reduction of white blood cells involving lymphopenia, reduction of macrophages in bone marrow, and augmented inflammatory responses to LPS. Dietary administration of the PFAAs also altered hepatic immune status by enhancing the number of intrahepatic immune cells, presumptive erythrocytes progenitors, and hepatic levels of erythropoietin.

Endocrine Disruption

The endocrine disruptive potentials of PFAAs have been summarized in a brief review [265]. In general, alterations of thyroid hormones and sex steroid hormones have been shown after exposure to primarily PFOS and PFOA, although PFDA-induced reductions of thyroid hormones have also been reported [266, 267]. Seacat *et al*. [268] first described alterations of circulating thyroid hormones in cynomolgus monkeys during chronic exposure to PFOS, which entailed significant reductions of triiodothyronine (T3) (by about 50%) that were greater and more consistent than those observed for total thyroxine (tT4, seen only in females) at serum levels of PFOS that reached 70–170 μg/ml. Values of thyroid-stimulating hormone (TSH) were quite variable and did not indicate compensatory elevation (by about twofold) until the end of the exposure period. This profile of primarily T3 reduction without an appreciative TSH response does not reflect classical hypothyroidism; rather, it resembles aspects of nonthyroidal illness syndrome, which is typically associated with a number of severe illnesses.

PFOS-induced alterations of thyroid hormones were confirmed in adult rat models [205, 214, 269]. However, in contrast to the monkeys, reductions of circulating tT4 were more pronounced and consistent than those of T3. These hormonal changes were abrupt. At an oral gavage dose that produced a serum PFOS level of 88 μg/ml, marked depressions of tT4 (by 50–75%) were seen within 1–3 days. In fact, it is interesting to note that thyroid hormones seem to be altered when serum PFOS level reaches the 70–90 μg/ml range, regardless of animal species (rat or monkey) or route of administration (diet, gavage, or drinking water), suggesting that PFOS effects on serum tT4 are directly related to

endogenous concentrations of the chemical. Furthermore, similar to the observation with monkeys, reductions of serum tT4 in rats failed to activate the hypothalamic–pituitary–thyroid (HPT) feedback mechanism to produce significant elevations of serum TSH.

A pronounced fall in serum tT4 with corresponding increases in TSH is typically noted during the course of pregnancy. Exposure of pregnant rats to PFOS exacerbated these hormonal shortfalls (both tT4 and T3) without further elevating the levels of TSH [214]. The effective dose of PFOS for tT4 reduction corresponded to maternal serum concentrations of 14–26 μg/ml (unpublished results). A similar effect of PFOS on serum tT4 was also seen in the pregnant mouse, although this rodent species appears to be less sensitive than the rat, with significant changes noted only at the doses that produced serum levels of 114–261 μg/ml [214, 270].

In utero exposure to PFOS led to postnatal mortality in the rat neonates, in a dose-dependent fashion [220]. Among the surviving pups, the ontogenetic increases of serum tT4 during the first 2 weeks of life were delayed or attenuated, with a lowest effective dose corresponding to serum PFOS levels of 60–72 μg/ml at 5 days of age and 30 μg/ml by 2 weeks. In contrast, only small changes were noted in the ontogenetic rises of T3 or TSH. Similar effects of PFOS on thyroid hormones in rats during development were also reported by Luebker *et al*. [221], where significant dose-related reductions of tT4 (46%) were noted on postnatal day 5 (serum PFOS level of 36 μg/ml). Consistent with the previous study, serum TSH remained unaltered. This lack of change in TSH was further corroborated by histological and morphometric evaluations of the fetal and neonatal thyroid glands, which indicated normal number and size distribution of follicles, as well as normal follicular epithelial cell height and colloid area, despite the PFOS-induced tT4 deficits [270]. In a cross-fostering study, Yu *et al*. [222] showed that pre- and postnatal exposure led to the most consistent effect of hypothyroxinemia and significant tT4 deficits were detected at rather low serum levels of PFOS (7–9 μg/ml). Although PFOS-related neonatal mortality was also observed in the mice, the ontogenetic increases of serum tT4 were not altered significantly in this species, a finding consistent with the relative insensitivity of mice to this chemical regarding thyroid hormone disruption [220].

In addition to the evaluation of PFOS effects on serum tT4, several studies have examined levels of circulating free T4 (fT4), the pool of hormone that is available for uptake by target cells and actions [214, 220, 221]. In these studies, fT4 was typically measured by analog radioimmunoassays (RIA) and reductions of free hormone produced by PFOS were similar to those observed in tT4. However, when the measurement of fT4 was carried out by including an equilibrium dialysis step prior to the standard RIA (ED-RIA), fT4 levels in the PFOS-treated rats were found to be comparable to those of controls [221]. Indeed, Chang *et al*. [271] further elaborated the merits of ED-RIA to eliminate the negative bias of fT4 determination produced by analog methods, primarily due to the high affinity for protein binding by PFOS. In light of these findings, the values of fT4 reported in previous PFOS studies may require reevaluation, and future investigations of these perfluorinated chemicals should employ this reference method.

Mechanisms underlying the PFOS-induced hypothyroxinemia are still under active investigation but do not likely involve altered *de novo* biosynthesis of the hormones or compromised integrity of the HPT axis. Yu *et al*. [269] reported no significant effects of PFOS on sodium iodide symporter gene expression (for iodide uptake) or thyroid peroxidase activity (for iodination of thyroglobulin and coupling into iodothyronine) in the thyroid gland. Chang *et al*. [272] showed that release of TSH from the pituitary in response to *ex vivo* TRH stimulation was not altered by PFOS exposure. In addition, when the hypothyroid drug propylthiouracil (PTU) was coadministered with PFOS, compensatory elevations of serum TSH that were equivalent to those elicited by PTU treatment alone were seen, indicating that the HPT axis in the PFOS-exposed rats was intact and fully functional. Importantly, in an acute exposure study, these investigators observed an abrupt fall of tT4, a transient increase in fT4 (determined by ED-RIA), and a corresponding transient decrease in TSH in circulation, accompanied by a brief increase in the expression of the gene for thyroid hormone-metabolizing enzyme UDP-glucuronosyltransferase 1A (UGT1A) in the liver, along with an increased urinary excretion of labeled tracer from ^{125}I-T4 over the course of 24 h following a single dose of PFOS. These findings are consistent with the hypothesis advanced by Gutshall *et al*. [267] with PFDA and suggest that PFOS may act by displacing thyroid hormones from their transport proteins in circulation. Indeed, this hypothesis was confirmed by Weiss *et al*. [273] who demonstrated that perfluorinated chemicals (including PFOS) are capable of competing with T4 and displacing hormone binding to the human thyroid hormone transport protein transthyretin (TTR). Hence, a plausible scenario can be constructed to account for the hypothyroxinemic effects of PFOS in the rats. PFOS in circulation competes with T4 and displaces the hormone from binding to TTR (the primary thyroid hormone transport protein in the rat), initially leading to a transient elevation of fT4 (within 6 h) and a brief compensatory decrease of TSH. Concomitantly, hepatic metabolism of the hormone by UGT1A is enhanced (presumably in response to the transient elevation of free hormone), which results in an increase of hormonal clearance and urinary excretion of iodide. As the fT4 level returns subsequently to normal (within 24 h), a new equilibrium is reached between normal complements of fT4 and TSH, but a net reduction of total T4 (resulted from protein-binding displacement and metabolism) ensues. A lack of significant change in TSH receptor gene expression in the thyroid gland is also consistent with the transient nature of change in TSH [269, 270]. Moreover, maintenance of fT4 levels is indirectly supported by a general lack of thyroid hormone-specific responses in the rat [219, 272, 274], suggesting that the functional thyroid status has not been compromised significantly by short-term exposure to the chemical. However, the biochemical and physiological sequelae derived from long-term displacement of T4 as a result of chronic PFOS exposure have not been vigorously investigated. Significant elevation of TSH in monkeys after 6 months of daily treatment with PFOS does raise the possibility of compensatory responses of the HPT axis after prolonged chemical exposure [268].

Effects of PFOA on thyroid hormones are generally not as well characterized as those of PFOS. Butenhoff *et al*. [164] evaluated the toxicity of PFOA in male

cynomolgus monkeys and reported that T3 was reduced significantly within 5 weeks of treatment when a serum level of 158 μg/ml was attained. Recovery of T3 deficits was noted upon cessation of PFOA exposure. Serum tT4, fT4, or TSH was not altered throughout the study. The preferential effects of PFOA on serum T3 and a lack of TSH compensatory response are similar to those observed with PFOS. Martin *et al*. [205] showed that serum tT4 and fT4 (measured by analog RIA) were markedly (by about 80%) and abruptly (1 day after oral gavage treatment) depressed by PFOA in adult male rats, while serum T3 was also reduced, though to a lesser extent (by 25%). In contrast, none of these thyroid hormones were affected by PFOA in mature female rats, primarily because these animals were able to clear the chemical effectively (Table 3), confirming that the endocrine disrupting effects of PFOA are directly related to endogenous accumulation of the chemical. PFOA may also act by displacing T4 from its binding protein, as the chemical has been shown to compete for binding to human TTR at a potency equivalent to that of PFOS [273]. Alternatively, based on a toxicogenomic analysis of rat liver after an acute exposure to PFOA, Martin *et al*. [205] suggested a possible role of peroxisome proliferators in the thyroid hormone imbalance, although this hypothesis has yet to be explored in detail.

In addition to thyroid hormone disruption, changes in sex steroid hormone biosynthesis by PFAAs have also been reported. Some of this information has been summarized previously [9]. In brief, PFOA has been shown to decrease serum and testicular testosterone and to increase serum estradiol in male rats, presumably via induction of hepatic aromatase [190, 275]. PFOS, PFOA, and telomer alcohols have been shown to exhibit estrogenic activity in cultured tilapia hepatocytes, yeast cells, and medaka hepatocytes [276–278] and to inhibit testicular steroidogenic enzymes [279, 341]. In addition, the long-chain PFAA perfluorododecanoic acid (PFdoDA) has recently been shown to decrease testosterone synthesis in male rats and to decrease serum estradiol and gene expression of estrogen receptors in the female rats, possibly through oxidative stress pathways [281–285].

Neurotoxicity

Slotkin *et al*. [286] characterized the neurotoxic potential of perfluorooctane sulfonamide (PFOSA), PFOS, PFBS, and PFOA in a neuronotypic PC12 cell model. PFOSA was found to enhance differentiation of cells into cholinergic and dopaminergic phenotypes, PFOS promoted the cholinergic phenotype at the expense of dopaminergic cells, PFBS suppressed differentiation of both phenotypes, and PFOA had little to no effect. Changes in synaptic transmission and inhibition of neurite outgrowth brought forth by PFOS were reported in cultured rat hippocampal neurons; the effects were more pronounced with PFSAs than PFCAs, and C8 being the optimum chain length [280, 287]. Subtle behavioral changes were noted in adult mice exposed to PFOS [288]. Expression of transcription factors, c-fos and c-jun, and calcium-dependent signals were altered in the hippocampus and cerebral cortex of

rats given PFOS [289]. However, after a single oral treatment of PFOS at doses (125–250 mg/kg) where convulsion was noted in rats and mice, no morphological changes were seen in the brain and changes of CNS neurotransmitter levels were not detected [290].

Although Butenhoff *et al*. [291] reported no significant developmental neurotoxicity associated with gestational and lactational exposure to PFOS, using the current testing guidelines, subtle effects have been shown in the brain after developmental exposure to PFAAs. Liu and colleagues have shown aberrant expression of genes involved in calcium signaling pathways, neuroactive ligand-receptor interactions, and long-term potentiation/depression in neonatal and adult brains exposed to PFOS during perinatal periods [292, 293]. Johansson *et al*. [294, 295] also demonstrated changes in proteins involved in neurogenesis and synaptogenesis in the developing mouse brain after neonatal exposure to PFOS or PFOA, which were accompanied by neurobehavioral defects in adulthood. Similar perturbed cognitive performance was also reported in an avian model after exposure to PFOS or PFOA *in ovo* [296]. Overall, investigation of PFAA neurotoxicity is only emerging. Because the blood–brain barrier is not completely closed to chemical trafficking until late in gestation (human) or postnatally (rodent), PFAAs may readily reach the immature brain to produce long-lasting effects. Hence, future work should focus on the developing nervous system to better explore the neurotoxic potential of these perfluorinated chemicals.

Modes of Action for PFAAs

A clear understanding of the key events involved in the mode of action (MOA) of an adverse outcome will be instrumental to health risk assessment of chemical exposure. Although the toxicities of PFAA exposure have been better characterized with animal models in the past decade, little progress has been made to clarify the MOA for these chemicals. The lone exception is activation of nuclear receptors by PFAAs, particularly PPARα, for which there is a preponderance of evidence. Wolf *et al*. [297] have compared the relative potency of various PFAAs using mouse and human PPARα reporter cell constructs, and their results are summarized in Table 4. In general, PFCAs are more active than PFSAs, the long-chain PFCAs (>C6) are more potent than the short-chain homologues, and mouse PPARα appears to be more sensitive than that of human. As discussed above, PPARα activation has been shown to be associated with carcinogenicity, hepatotoxicity, developmental toxicity, immunotoxicity, and perhaps even endocrine disruption in laboratory rodents. In fact, key events of the PPARα pathway may play a critical role in the interpretation of PFAA-induced tumors observed in the rodent model, as expert panels have previously surmised that this mode of action is not likely to be relevant for humans [298, 299]. Recent studies using humanized PPARα mice also supported this species difference [198, 300]. However, this assertion has recently been challenged [301], and a final verdict for human relevance of the PFAA-related tumor induction

Table 4 Comparative potency of PFAAs for PPARα [297]

Compound	C_{20max} (μM)	
	Mouse	Human
PFNA (C9)	5	11
PFOA (C8)	6	16
PFDA (C10)	20	No activity
PFHxA (C6)	38	47
PFBA (C4)	51	75
PFHxS (C6)	76	81
PFOS (C8)	94	262
PFBS (C4)	317	206

must await further clarification. In the same vein, the relevance of other PFAA-evoked, PPARα-dependent effects (such as disruption of lipid metabolism, hepatotoxicity, developmental toxicity, and immunotoxicity) to human health risks will require additional scrutiny. In addition to PPARα and other nuclear receptor pathways, several possible mechanisms for PFAA toxicity have been suggested. These include oxidative stress [253, 286, 302–304], effects on other cell signaling pathways [252, 305, 306], and epigenetic changes [307]. Other putative mechanisms undoubtedly will emerge as investigation in this area intensifies in the future.

Epidemiology

Occupational biomonitoring studies have been conducted for PFAAs over the past several decades. Olsen and colleagues reported a lack of changes in serum hepatic enzymes, cholesterol, lipoproteins, or thyroid hormones associated with serum PFOS levels less than 6 μg/ml in the fluorochemical production plant workers (only few individuals had levels greater than 6 μg/ml) [122, 123]. Little change in mortality rate was seen in production workers, although the risk of death from bladder cancer was increased (with only three cases reported) [308]. Further analysis with larger cohorts of all living current and former employees did not support an association between bladder or other cancers and PFOS exposure [309, 310]. In fact, examination of health claim data (episodes of care) showed that illness and disorders reported among workers in the PFOS production plant were comparable to that of the non-PFOS-related work forces [311]. These investigators have also extended their epidemiological examination to PFOA occupational exposure and reported no significant associations between serum PFOA and reproductive hormones in men [121], serum cholesterol, or low-density lipoprotein; although high-density lipoprotein and free T4 were negatively associated with PFOA, triglycerides and T3 tended to be positively associated. Several explanations were offered by these authors to account for the inconsistent and marginal changes observed [313]. Results from a mortality study showed no association between PFOA exposure and liver, pancreatic, or testicular cancer (a tumor triad seen in

rodent models) in the production workers, but an inconsistent association was noted with prostate cancer, cardiovascular disease, and diabetes [314]. In reviewing 30 years of medical surveillance of PFOA production workers, Costa *et al*. [315] concluded that no specific clinical disease was associated with exposure to the fluorochemical, and biochemical parameters reflecting hepatic, renal, and hormonal functions appeared to be within reference ranges; however, a significant association of serum cholesterol and uric acid with PFOA was evident, indicating that further investigation of PFOA influences on intermediary metabolism is warranted. Based on the available information, Butenhoff *et al*. [316] provided a health risk characterization of PFOA exposure for the general population and suggested a wide "margin of exposure" that would represent a substantial protection of children, adult, and the elderly. Similarly, an epidemiological study of workers exposed to surfactant containing PFNA for more than a decade has been conducted, and no adverse clinical effects were detected from occupational exposure to this fluorochemical [317].

Prompted by the toxicity findings in animal models, a myriad of epidemiological investigations in general population have been launched over the past 5 years. The reproductive and developmental effects of PFOS and PFOA have by far attracted the most attention. Examining "time-to-pregnancy" among 1,240 pregnant women in the Danish National Birth Cohort from 1996 to 2002, Fei *et al*. [318] suggested that PFOA and PFOS exposure might be associated with a reduction of fecundity. Fetal growth, birth weight, and size have been negatively associated with maternal blood levels of both PFOS and PFOA in several cohort studies [139, 319, 320], although absence of such effects has also been reported in other studies [321–323]. Stein *et al*. [324] examined self-reported pregnancy outcomes in Mid-Ohio Valley residents between 2000 and 2006 (2,000–5,000 cases) and identified modest associations of PFOA with preeclampsia and birth defects and of PFOS with preeclampsia and low birth weight. Nolan *et al*. [325] evaluated a smaller Ohioan cohort exposed to PFOA-contaminated drinking water and found that PFOA was associated with maternal anemia and dysfunctional labor but not with congenital anomalies or delivery complication. The strengths and weaknesses of these studies and interpretations of their findings have been addressed in a thorough review [13]. Follow-up evaluations of infants and children in the Danish National Birth Cohort indicated no associations between prenatal exposure to PFAAs and risk of infectious diseases, developmental milestones, and behavioral and motor coordination problems [326–328]. A recent British cohort study also did not find an association between maternal PFAA exposure and altered age at menarche of their offspring [329].

The "C8 Health Project" was launched to investigate the potential health effects of exposure to PFOA from drinking water in the Mid-Ohio Valley areas. Associations of PFOS and PFOA with serum lipids and uric acid were reported among the local residents, although those with type II diabetes were not indicated [330–333]. A number of exploratory cross-sectional studies analyzing NHANES results have also been conducted. Lin *et al*. [334, 335] suggested that serum PFAAs were associated with altered glucose homeostasis, indicators of metabolic syndrome,

and elevated liver enzymes (particularly in obese subjects); Nelson *et al*. [336] indicated a positive association between serum PFAAs and cholesterol; Melzer *et al*. [337] showed a significant association of PFOS and PFOA with thyroid disease; and Hoffman *et al*. [338] reported an increased odds ratio of attention deficit hyperactivity disorder with higher serum PFAA levels. Typically, the odds ratios for these clinical disorders range from 1 to 2, although the trends are statistically significant. In light of the structural resemblance of PFAAs to fatty acids and their biochemical actions on PPAR pathways, iterative research with animal models to better elucidate the effects of these fluorochemicals on intermediary metabolism is a logical next step. On the other hand, in Danish cohorts, high PFAA levels were associated with fewer normal sperm [339], but no association was found with risk of prostate, bladder, or liver cancer in this population [340]. Steenland *et al*. [14] recently reviewed the epidemiological literature for PFOA and noted that available data were insufficient to draw firm conclusions regarding the role of fluorochemicals for any of the diseases of concern.

Summary

Since a smattering of papers on PFAAs first appeared in the literature before the turn of the century, there has been an explosion of studies on these chemicals just in the last 5 years. This chapter provides a summary of our current understanding of PFAA exposure in the environment and in human populations, their toxicological profiles in laboratory animals, and epidemiological findings in general and targeted populations. Improved sensitivity and reproducibility of analytical methods to readily detect multiple PFAAs at the parts per trillion level have afforded cross-study comparisons and the ability to track changes in trends. Continuous biomonitoring studies should provide updates regarding changes in PFAA exposure in the future. These changes in exposure are likely to occur as PFAAs in commerce (such as the C8 chemicals) are replaced by the short-chain homologues or entirely different chemistries. Descriptive characterization of the overt toxicity of PFAAs (particularly the long-chain homologues) in animal models should open the door for further investigation of the more subtle biochemical and physiological perturbations potentially elicited by these chemicals. These combined advances will facilitate an informed and reliable risk assessment of human and environmental health for these perfluorinated chemicals. However, two issues must be considered in extrapolating the data from animal studies to human health risks. As shown in Table 3, the accumulation of these chemicals varies tremendously between congeners of different chain length and functional group, and most importantly, the species differences between rodents and humans are profound. Simple correction factors will not be sufficient or appropriate to address these differences. Rather, a better understanding of the cellular and molecular mechanisms (such as the involvement of transporters, i.e. OATs) that control the clearance of these chemicals as well as possible homology between species is needed. Secondly, as indicated

by NHANES and numerous other monitoring studies, multiple PFAAs are detected in human and wildlife populations, and the profile of PFAA exposure is expected to change with time. Thus, the combined health risks of a mixture of these chemicals must be considered. In closing, many discoveries have been made with this intriguing family of chemicals in the past decade, but much more information will be needed to ascertain their adverse health effects.

Acknowledgments The information in this document has been subjected to review by the Office of Pollution Prevention and Toxics and the Office of Research and Development of US Environmental Protection Agency and approved for publication. Approval does not signify that the contents reflect the views of the agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

References

1. D'eon JC, Crozier PW, Furdui CI, Reiner EJ, Libelo EL, Mabury SA (2009) Perfluorinated phosphonic acids in Canadian surface waters and wastewater treatment plant effluent: discovery of a new class of perfluorinated acids. Environ Toxicol Chem 28:2101–2107
2. D'eon JC, Mabury SA (2010) Uptake and eliminated of perfluorinated phosphonic acids in the rat. Environ Toxicol Chem 29:1319–1329
3. Giesy JP, Kannan K (2001) Perfluorochemical surfactants in the environment. Environ Sci Technol 36:146A–152A
4. Kudo N, Kawashima Y (2003) Toxicity and toxicokinetics of perflurooctanoic acid in humans and animals. J Toxicol Sci 28:49–57
5. Kennedy GL, Butenhoff JL, Olsen GW, O'Connor JC, Seacat AM, Perkins RG, Biegel LB, Murphy SR, Farrar DG (2004) The toxicology of perfluorooctanoate. Crit Rev Toxicol 34:351–384
6. Lau C, Butenhoff JL, Rogers JM (2004) The developmental toxicity of perfluoroalkyl acids and their derivatives. Toxicol Appl Pharmacol 198:231–241
7. Houde M, Martin JW, Letcher RJ, Solomon KR, Muir DCG (2006) Biological monitoring of polyfluoroalkyl substances: a review. Environ Sci Technol 40:3463–3473
8. Beach SA, Newsted JL, Coady K, Giesy JP (2006) Ecotoxicological evaluation of perfluorooctanesulfonate (PFOS). Rev Environ Contam Toxicol 186:133–174
9. Lau C, Anitole K, Hodes C, Lai D, Pfahles-Hutchens A, Seed J (2007) Perfluoroalkyl acids: a review of monitoring and toxicological findings. Toxicol Sci 99:366–394
10. Andersen ME, Butenhoff JL, Chang SC, Farrar DG, Kennedy GL, Lau C, Olsen GW, Seed J, Wallace KB (2008) Perfluoroalkyl acids and related chemistries – Toxicokinetics and modes of action. Toxicol Sci 102:3–14
11. Kovarova J, Svobodova Z (2008) Perfluorinated compounds: occurrence and risk profile. Neuroendocrinol Lett 29:599–608
12. Fromme H, Tittlemier SA, Völkel W, Wilhelm M, Twardella D (2009) Perfluorinated compounds – exposure assessment for the general population in Western countries. Int J Hyg Environ Health 212:239–270
13. Olsen GW, Butenhoff JL, Zobel LR (2009) Perfluoroalkyl chemicals and human fetal development: an epidemiologic review with clinical and toxicological perspectives. Reprod Toxicol 27:212–230
14. Steenland K, Fletcher T, Savitz DA (2010) Epidemiologic evidence on the health effects of perfluorooctanoic acid (PFOA). Environ Health Perspect 118:1100–1108

15. Giesy JP, Naile JE, Khim JS, Jones PD, Newsted JL (2010) Aquatic toxicology of perfluorinated chemicals. Rev Environ Contam Toxicol 202:1–52
16. Lehmler HJ (2005) Synthesis of environmentally relevant fluorinated surfactants – a review. Chemosphere 58:1471–1496
17. Kissa E (2001) Fluorinated surfactants and repellants, 2nd edn. Marcel Decker, New York, NY
18. Vanden Heuvel JP, Sterchele PE, Nesbit DJ, Peterson RE (1993) Coordinate induction of acyl-CoA binding protein, fatty acid binding protein and peroxisomal β-oxidation by peroxisome proliferators. Biochim Biophys Acta 1177:183–190
19. Luebker DJ, Hansen KJ, Bass NM, Butenhoff JL, Seacat AM (2002) Interactions of fluorochemicals with rat liver fatty acid-binding protein. Toxicology 176:175–185
20. Woodcroft MW, Ellis DA, Rafferty SP, Burns DC, March RE, Stock NL, Trumpour KS, Yee J, Munro K (2010) Experimental characterization of the mechanism of perfluorocarboxylic acids' liver protein bioaccumulation: the key role of the neutral species. Environ Toxicol Chem 29:1669–1677
21. Vanden Heuvel JP, Kuslikis BI, Peterson RE (1992) Covalent binding of perfluorinated fatty acids to proteins in the plasma, liver and testes of rats. Chem Biol Interact 82:317–328
22. Han X, Snow TA, Kemper RA, Jepson GW (2003) Binding of perfluorooctanoic acid to rat and human plasma proteins. Chem Res Toxicol 16:775–781
23. Han X, Hinderliter PM, Snow TA, Jepson GW (2004) Binding of perfluorooctanoic acid to rat liver-form and kidney-form α2u-globulins. Drug Chem Toxicol 27:341–360
24. Jones PD, Hu W, De Coen W, Newsted JL, Giesy JP (2003) Binding of perfluorinated fatty acids to serum proteins. Environ Toxicol Chem 22:2639–2649
25. Chen YM, Guo LH (2009) Fluorescence study on site-specific binding of perfluoroalkyl acids to human serum albumin. Arch Toxicol 83:255–261
26. Bischel HN, Macmanus-Spencer LA, Luthy RG (2010) Noncovalent interactions of long-chain perfluoroalkyl acids with serum albumin. Environ Sci Technol 44:5263–5269
27. Weaver YM, Ehresman DJ, Butenhoff JL, Hagenbuch B (2010) Roles of rat renal organic anion transporters in transporting perfluorinated carboxylates with different chain lengths. Toxicol Sci 113:305–314
28. Cheng X, Klaassen CD (2008) Critical role of PPARα in perfluorooctanoic acid- and perfluorodecanoic acid-induced downregulation of Oatp uptake transporters in mouse livers. Toxicol Sci 106:37–45
29. Ikeda T, Aiba K, Fukuda K, Tanaka M (1985) The induction of peroxisome proliferation in rat liver by perfluorinated fatty acids, metabolically inert derivatives of fatty acids. J Biochem 98:475–482
30. Pastoor TP, Lee KP, Perri MA, Gillies PJ (1987) Biochemical and morphological studies of ammonium perfluorooctanoate-induced hepatomegaly and peroxisome proliferation. Exp Mol Pathol 47:98–109
31. Goecke-Flora CM, Wyman JF, Jarnot BM, Reo NV (1995) Effect of the peroxisome proliferator perfluoro-n-decanoic acid on glucose transport in the isolated perfused rat liver. Chem Res Toxicol 8:77–81
32. Intrasuksri U, Rangwala SM, O'Brien M, Noonan DJ, Feller DR (1998) Mechanisms of peroxisome proliferation by perfluorooctanoic acid and endogenous fatty acids. Gen Pharmacol 31:187–197
33. Vanden Heuvel JP, Thompson JT, Frame SR, Gillies PJ (2006) Differential activation of nuclear receptors by perfluorinated fatty acid analogs and natural fatty acids: a comparison of human, mouse, and rat peroxisome proliferator-activated receptor-α, -β, and -γ, liver X receptor-β, and retinoid X receptor-α. Toxicol Sci 92:476–489
34. Takacs ML, Abbott BD (2007) Activation of mouse and human peroxisome proliferator-activated receptors (α, β/δ, γ) by perfluorooctanoic acid and perfluorooctane sulfonate. Toxicol Sci 95:108–117
35. Rosen MB, Lee JS, Ren H, Vallanat B, Liu J, Waalkes MP, Abbott BD, Lau C, Corton JC (2008) Toxicogenomic dissection of the perfluorooctanoic acid transcript profile in mouse

liver: evidence for the involvement of nuclear receptors PPARα and CAR. Toxicol Sci 103:46–56
36. Rosen MB, Abbott BD, Wolf DC, Corton JC, Wood CR, Schmid JE, Das KP, Zehr RD, Blair ET, Lau C (2008) Gene profiling in the livers of wild-type and PPARα-null mice exposed to perfluorooctanoic acid. Toxicol Pathol 36:592–607
37. Cheng X, Klaassen CD (2008) Perfluorocarboxylic acids induce cytochrome P450 enzymes in mouse liver through activation of PPAR-α and CAR transcription factors. Toxicol Sci 106:29–36
38. Ren H, Vallanat B, Nelson DM, Yeung LW, Guruge KS, Lam PK, Lehman-McKeeman LD, Corton JC (2009) Evidence for the involvement of xenobiotic-responsive nuclear receptors in transcriptional effects upon perfluoroalkyl acid exposure in diverse species. Reprod Toxicol 27:266–277
39. Hu W, Jones PD, DeCoen W, King L, Fraker P, Newsted J, Giesy JP (2003) Alterations in cell membrane properties caused by perfluorinated compounds. Comp Biochem Physiol C Toxicol Pharmacol 135:77–88
40. Lehmler HJ, Xie W, Bothun GD, Bummer PM, Knutson BL (2006) Mixing of perfluorooctanesulfonic acid (PFOS) potassium salt with dipalmitoyl phosphatidylcholine (DPPC). Colloids Surf B Biointerfaces 51:25–29
41. Xie W, Kania-Korwel I, Bummer PM, Lehmler HJ (2007) Effect of potassium perfluorooctanesulfonate, perfluorooctanoate and octanesulfonate on the phase transition of dipalmitoylphosphatidylcholine (DPPC) bilayers. Biochim Biophys Acta 1768:1299–1308
42. Matyszewska D, Leitch J, Bilewicz R, Lipkowski J (2008) Polarization modulation infrared reflection-absorption spectroscopy studies of the influence of perfluorinated compounds on the properties of a model biological membrane. Langmuir 24:7408–7412
43. Kawamoto K, Nishikawa Y, Oami K, Jin Y, Sato I, Saito N, Tsuda S (2008) Effects of perfluorooctane sulfonate (PFOS) on swimming behavior and membrane potential of paramecium caudatum. J Toxicol Sci 33:155–161
44. Xie W, Ludewig G, Wang K, Lehmler HJ (2010) Model and cell membrane partitioning of perfluorooctanesulfonate is independent of the lipid chain length. Colloids Surf B Biointerfaces 76:128–136
45. Xie W, Bothun GD, Lehmler HJ (2010) Partitioning of perfluorooctanoate into phosphatidylcholine bilayers is chain length-independent. Chem Phys Lipids 163:300–308
46. Harada KH, Ishii TM, Takatsuka K, Koizumi A, Ohmori H (2006) Effects of perfluorooctane sulfonate on action potentials and currents in cultured rat cerebellar Purkinje cells. Biochem Biophys Res Commun 351:240–245
47. Matyszewska D, Leitch J, Bilewicz R, Lipkowski J (2008) Polarization modulation infrared reflection-absorption spectroscopy studies of the influence of perfluorinated compounds on the properties of a model biological membrane. Langmuir 24:7408–7412
48. Upham BL, Deocampo ND, Wurl B, Trosko JE (1998) Inhibition of gap junctional intercellular communication by perfluorinated fatty acids is dependent on the chain length of the fluorinated tail. Int J Cancer 78:491–495
49. Hu W, Jones PD, Upham BL, Trosko JE, Lau C, Giesy JP (2002) Inhibition of gap junctional intercellular communication by perfluorinated compounds in rat liver and dolphin kidney epithelial cell lines *in vitro* and Sprague–Dawley rats *in vivo*. Toxicol Sci 68:429–436
50. Upham BL, Park JS, Babica P, Sovadinova I, Rummel AM, Trosko JE, Hirose A, Hasegawa R, Kanno J, Sai K (2009) Structure-activity-dependent regulation of cell communication by perfluorinated fatty acids using *in vivo* and *in vitro* model systems. Environ Health Perspect 117:545–551
51. Berthiaume J, Wallace KB (2002) Perfluorooctanoate, perflourooctanesulfonate, and N-ethyl perfluorooctanesulfonamido ethanol; Peroxisome proliferation and mitochondrial biogenesis. Toxicol Lett 129:23–32
52. O'Brien TM, Wallace KB (2004) Mitochondrial permeability transition as the critical target of *N*-acetyl perfluorooctane sulfonamide toxicity *in vitro*. Toxicol Sci 82:333–340

53. Walters MW, Bjork JA, Wallace KB (2009) Perfluorooctanoic acid stimulated mitochondrial biogenesis and gene transcription in rats. Toxicology 264:10–15
54. Kleszczyński K, Stepnowski P, Składanowski AC (2009) Mechanism of cytotoxic action of perfluorinated acids II. Disruption of mitochondrial bioenergetics. Toxicol Appl Pharmacol 235:182–190
55. Gordon SC (2010) Toxicological evaluation of ammonium 4,8-dioxa-3*H*-perfluorononanoate, a new emulsifier to replace ammonium perfluorooctanoate in fluoropolymer manufacturing. Regul Toxicol Pharmacol 59:64–80
56. Paul AG, Jones KC, Sweetman AJ (2009) A first global production, emission, and environmental inventory for perfluorooctane sulfonate. Environ Sci Technol 43:386–392
57. Wang N, Szostek B, Buck RC, Folsom PW, Sulecki LM, Capka V, Berti WR, Gannon JT (2005) Fluorotelomer alcohol biodegradation-direct evidence that perfluorinated carbon chains breakdown. Environ Sci Technol 39:7516–7528
58. Wang N, Szostek B, Folsom PW, Sulecki LM, Capka V, Buck RC, Berti WR, Gannon JT (2005) Aerobic biotransformation of ^{14}C-labeled 8-2 telomer B alcohol by activated sludge from a domestic sewage treatment plant. Environ Sci Technol 39:531–538
59. Wallington TJ, Hurley MD, Xia J, Wuebbles DJ, Sillman S, Ito A, Penner JE, Ellis DA, Martin J, Mabury SA, Nielsen OJ, Sulbaek Andersen MP (2006) Formation of C7F15COOH (PFOA) and other perfluorocarboxylic acids during the atmospheric oxidation of 8:2 fluorotelomer alcohol. Environ Sci Technol 40:924–930
60. Liu J, Lee LS, Nies LF, Nakatsu CH, Turco RF (2007) Biotransformation of 8:2 fluorotelomer alcohol in soil and by soil bacteria isolates. Environ Sci Technol 41:8024–8030
61. Wang N, Szostek B, Buck RC, Folsom PW, Sulecki LM, Gannon JT (2009) 8:2 fluorotelomer alcohol aerobic soil biodegradation: pathways, metabolites, and metabolite yields. Chemosphere 75:1089–1096
62. Arakaki A, Ishii Y, Tokuhisa T, Murata S, Sato K, Sonoi T, Tatsu H, Matsunaga T (2010) Microbial biodegradation of a novel fluorotelomer alcohol, 1*H*,1*H*,2*H*,2*H*,8*H*,8*H*-perfluorododecanol, yields short fluorinated acids. Appl Microbiol Biotechnol 88:1193–1203
63. Young CJ, Mabury SA (2010) Atmospheric perfluorinated acid precursors: chemistry, occurrence, and impacts. Rev Environ Contam Toxicol 208:1–109
64. Lee H, D'eon J, Mabury SA (2010) Biodegradation of polyfluoroalkyl phosphates as a source of perfluorinated acids to the environment. Environ Sci Technol 44:3305–3310
65. Busch J, Ahrens L, Sturm R, Ebinghaus R (2010) Polyfluoroalkyl compounds in landfill leachates. Environ Pollut 158:1467–1471
66. Washington JW, Yoo H, Ellington JJ, Jenkins TM, Libelo EL (2010) Concentrations, distribution, and persistence of perfluoroalkylates in sludge-applied soils near Decatur, Alabama, USA. Environ Sci Technol 44:8390–8396
67. Yoo H, Washington JW, Ellington JJ, Jenkins TM, Neill MP (2010) Concentrations, distribution, and persistence of fluorotelomer alcohols in sludge-applied soils near Decatur, Alabama, USA. Environ Sci Technol 44:8397–8402
68. Nabb DL, Szostek B, Himmelstein MW, Mawn MP, Gargas ML, Sweeney LM, Stadler JC, Buck RC, Fasano WJ (2007) *In vitro* metabolism of 8-2 fluorotelomer alcohol: interspecies comparisons and metabolic pathway refinement. Toxicol Sci 100:333–344
69. Fasano WJ, Sweeney LM, Mawn MP, Nabb DL, Szostek B, Buck RC, Gargas ML (2009) Kinetics of 8-2 fluorotelomer alcohol and its metabolites, and liver glutathione status following daily oral dosing for 45 days in male and female rats. Chem Biol Interact 180: 281–295
70. Liu J, Wang N, Szostek B, Buck RC, Panciroli PK, Folsom PW, Sulecki LM, Bellin CA (2010) 6-2 Fluorotelomer alcohol aerobic biodegradation in soil and mixed bacterial culture. Chemosphere 78:437–444
71. Myers AL, Mabury SA (2010) Fate of fluorotelomer acids in a soil-water microcosm. Environ Toxicol Chem 29:1689–1695
72. Stahl T, Heyn J, Thiele H, Hüther J, Failing K, Georgii S, Brunn H (2009) Carryover of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) from soil to plants. Arch Environ Contam Toxicol 57:289–298

73. Armitage JM, Schenker U, Scheringer M, Martin JW, Macleod M, Cousins IT (2009) Modeling the global fate and transport of perfluorooctane sulfonate (PFOS) and precursor compounds in relation to temporal trends in wildlife exposure. Environ Sci Technol 43:9274–9280
74. Stock NL, Furdui VI, Muir DC, Mabury SA (2007) Perfluoroalkyl contaminants in the Canadian Arctic: evidence of atmospheric transport and local contamination. Environ Sci Technol 41:3529–3536
75. Young CJ, Furdui VI, Franklin J, Koerner RM, Muir DC, Mabury SA (2007) Perfluorinated acids in Arctic snow: new evidence for atmospheric formation. Environ Sci Technol 41:3455–3461
76. Yarwood G, Kemball-Cook S, Keinath M, Waterland RL, Korzeniowski SH, Buck RC, Russell MH, Washburn ST (2007) High-resolution atmospheric modeling of fluorotelomer alcohols and perfluorocarboxylic acids in the North American troposphere. Environ Sci Technol 41:5756–5762
77. Kwok KY, Taniyasu S, Yeung LW, Murphy MB, Lam PK, Horii Y, Kannan K, Petrick G, Sinha RK, Yamashita N (2010) Flux of perfluorinated chemicals through wet deposition in Japan, the United States, and several other countries. Environ Sci Technol 44:7043–7049
78. Yamashita N, Kannan K, Taniyasu S, Horii Y, Petrick G, Gamo T (2005) A global survey of perfluorinated acids in oceans. Mar Pollut Bull 51:658–668
79. Yamashita N, Taniyasu S, Petrick G, Wei S, Gamo T, Lam PK, Kannan K (2008) Perfluorinated acids as novel chemical tracers of global circulation of ocean waters. Chemosphere 70:1247–1255
80. Busch J, Ahrens L, Xie Z, Sturm R, Ebinghaus R (2010) Polyfluoroalkyl compounds in the East Greenland Arctic Ocean. J Environ Monit 12:1242–1246
81. Ahrens L, Xie Z, Ebinghaus R (2010) Distribution of perfluoroalkyl compounds in seawater from northern Europe, Atlantic Ocean, and Southern Ocean. Chemosphere 78:1011–1016
82. Davis KL, Aucoin MD, Larsen BS, Kaiser MA, Hartten AS (2007) Transport of ammonium perfluorooctanoate in environmental media near a fluoropolymer manufacturing facility. Chemosphere 67:2011–2019
83. Harada K, Nakasanishi S, Sasaki K, Furuyama K, Nakayama S, Saito N, Yamakawa K, Koizumi A (2006) Particle size distribution and respiratory deposition estimates of airborne perfluorooctanoate and perfluooctanesulfonate in Kyoto area, Japan. Bull Environ Contam Toxicol 76:306–310
84. Barber JL, Berger U, Chaemfa C, Huber S, Jahnke A, Temme C, Jones KC (2007) Analysis of per- and polyfluorinated alkyl substances in air samples from Northwest Europe. J Environ Monit 9:530–541
85. Niisoe T, Harada KH, Ishikawa H, Koizumi A (2009) Long-term simulation of human exposure to atmospheric perfluorooctanoic acid (PFOA) and perfluorooctanoate (PFO) in the Osaka urban area, Japan. Environ Sci Technol 44:7852–7857
86. Langer V, Dreyer A, Ebinghaus R (2010) Polyfluorinated compounds in residential and nonresidential indoor air. Environ Sci Technol 44:8075–8081
87. Strynar MJ, Lindstrom AB (2008) Perfluorinated compounds in house dust from Ohio and North Carolina, USA. Environ Sci Technol 42:3751–3756
88. Kato K, Calafat AM, Needham LL (2009) Polyfluoroalkyl chemicals in house dust. Environ Res 109:518–523
89. Björklund JA, Thuresson K, De Wit CA (2009) Perfluoroalkyl compounds (PFCs) in indoor dust: concentrations, human exposure estimates, and sources. Environ Sci Technol 43: 2276–2281
90. Wang Y, Fu J, Wang T, Liang Y, Pan Y, Cai Y, Jiang G (2010) Distribution of perfluorooctane sulfonate and other perfluorochemicals in the ambient environment around a manufacturing facility in China. Environ Sci Technol 44:8062–8067
91. Rumsby PC, McLaughlin CL, Hall T (2009) Perfluorooctane sulphonate and perfluorooctanoic acid in drinking and environmental waters. Philos Transact A Math Phys Eng Sci 367:4119–4136

92. Suja F, Pramanik BK, Zain SM (2009) Contamination, bioaccumulation and toxic effects of perfluorinated chemicals (PFCs) in the water environment: a review paper. Water Sci Technol 60:1533–1544
93. Mak YL, Taniyasu S, Yeung LW, Lu G, Jin L, Yang Y, Lam PK, Kannan K, Yamashita N (2009) Perfluorinated compounds in tap water from China and several other countries. Environ Sci Technol 43:4824–4829
94. Quiñones O, Snyder SA (2009) Occurrence of perfluoroalkyl carboxylates and sulfonates in drinking water utilities and related waters from the United States. Environ Sci Technol 43:9089–9095
95. Ericson I, Domingo JL, Nadal M, Bigas E, Llebaria X, van Bavel B, Lindström G (2009) Levels of perfluorinated chemicals in municipal drinking water from Catalonia, Spain: public health implications. Arch Environ Contam Toxicol 57:631–638
96. Jin YH, Liu W, Sato I, Nakayama SF, Sasaki K, Saito N, Tsuda S (2009) PFOS and PFOA in environmental and tap water in China. Chemosphere 77:605–611
97. Nakayama SF, Strynar MJ, Reiner JL, Delinsky AD, Lindstrom AB (2010) Determination of perfluorinated compounds in the upper Mississippi river basin. Environ Sci Technol 44:4103–4109
98. Paustenbach DJ, Panko JM, Scott PK, Unice KM (2007) A methodology for estimating human exposure to perfluorooctanoic acid (PFOA): a retrospective exposure assessment of a community (1951-2003). J Toxicol Environ Health A 70:28–57
99. Drinking Water Inspectorate (2007) Guidance on the water supply (water quality) regulation 2000–2001 specific to PFOS (perfluorooctane sulphonate) and PFOA (perfluorooctanoic acid) concentrations in drinking water
100. Drinking Water Commission, German Ministry of Health (2006) Provisional evaluation of PFT in drinking water with the guide substances perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) as examples. Online available at http://www.umweltbundesamt.de/uba-info-presse-e/hintergrund/pft-in-drinking-water.pdf
101. New Jersey health-based drinking water guidance for PFOA (2009). Online available at http://www.state.nj.us/dep/watersupply/pfoa.htm
102. Minnesota health-based drinking water guidance for perfluorinated chemicals (2009). Online available at http://www.health.state.mn.us/divs/eh/risk/guidance/gw/table.html
103. US Environmental Protection Agency (2009) Provisional health advisory for PFOA and PFOS in drinking water. Online available at http://water.epa.gov/action/advisories/drinking/upload/2009_01_15_criteria_drinking_pha-PFOA_PFOS.pdf
104. Wilhelm M, Kraft M, Rauchfuss K, Hölzer J (2008) Assessment and management of the first German case of a contamination with perfluorinated compounds (PFC) in the Region Sauerland, North Rhine-Westphalia. J Toxicol Environ Health A 71:725–733
105. Brede E, Wilhelm M, Göen T, Müller J, Rauchfuss K, Kraft M, Hölzer J (2010) Two-year follow-up biomonitoring pilot study of residents' and controls' PFC plasma levels after PFOA reduction in public water system in Arnsberg, Germany. Int J Hyg Environ Health 213:217–223
106. Renner R (2009) Are perfluorochemicals widespread in biosolids? Environ Sci Technol 43:5164
107. Clarke BO, Smith SR (2011) Review of 'emerging' organic contaminants in biosolids and assessment of international research priorities for the agricultural use of biosolids. Environ Int 37:226–247
108. Bossi R, Strand J, Sortkjaer O, Larsen MM (2007) Perfluoroalkyl compounds in Danish wastewater treatment plants and aquatic environments. Environ Int 34:443–450
109. Plumlee MH, Larabee J, Reinhard M (2008) Perfluorochemicals in water reuse. Chemosphere 72:1541–1547
110. Ahrens L, Felizeter S, Sturm R, Xie Z, Ebinghaus R (2009) Polyfluorinated compounds in waste water treatment plant effluents and surface waters along the River Elbe, Germany. Mar Pollut Bull 58:1326–1333
111. Yu J, Hu J, Tanaka S, Fujii S (2009) Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) in sewage treatment plants. Water Res 43:2399–2408

112. Guo R, Sim WJ, Lee ES, Lee JH, Oh JE (2010) Evaluation of the fate of perfluoroalkyl compounds in wastewater treatment plants. Water Res 44:3476–3486
113. Kannan K, Koistinen J, Beckmen K, Evans T, Gorzelany JF, Hansen KJ, Jones PD, Helle E, Nyman M, Giesy JP (2001) Accumulation of perfluorooctane sulfonate in marine mammals. Environ Sci Technol 35:1593–1598
114. Giesy JP, Kannan K (2001) Global distribution of perfluorooctane sulfonate in wildlife. Environ Sci Technol 35:1339–1342
115. Giesy JP, Kannan K, Jones PD (2001) Global biomonitoring of perfluorinated organics. Sci World J 1:627–629
116. Löfstrand K, Jörundsdóttir H, Tomy G, Svavarsson J, Weihe P, Nygård T, Bergman K (2008) Spatial trends of polyfluorinated compounds in guillemot (Uria aalge) eggs from North-Western Europe. Chemosphere 72:1475–1480
117. Ahrens L, Siebert U, Ebinghaus R (2009) Temporal trends of polyfluoroalkyl compounds in harbor seals (*Phoca vitulina*) from the German Bight, 1999-2008. Chemosphere 76:151–518
118. Hart K, Gill VA, Kannan K (2009) Temporal trends (1992-2007) of perfluorinated chemicals in Northern Sea Otters (*Enhydra lutris kenyoni*) from South-Central Alaska. Arch Environ Contam Toxicol 56:607–614
119. Butt CM, Berger U, Bossi R, Tomy GT (2010) Levels and trends of poly- and perfluorinated compounds in the arctic environment. Sci Total Environ 408:2936–2965
120. Letcher RJ, Bustnes JO, Dietz R, Jenssen BM, Jørgensen EH, Sonne C, Verreault J, Vijayan MM, Gabrielsen GW (2010) Exposure and effects assessment of persistent organohalogen contaminants in arctic wildlife and fish. Sci Total Environ 408:2995–3043
121. Olsen GW, Gilliland FD, Burlew MM, Burris JM, Mandel JS, Mandel JH (1998) An epidemiologic investigation of reproductive hormones in men with occupational exposure to perfluorooctanoic acid. J Occup Environ Med 40:614–622
122. Olsen GW, Burris JM, Mandel JH, Zobel LR (1999) Serum perfluorooctane sulfonate and hepatic and lipid clinical chemistry tests in fluorochemical production employees. J Occup Environ Med 41:799–806
123. Olsen GW, Burris JM, Burlew MM, Mandel JH (2003) Epidemiologic assessment of worker serum perfluorooctanesulfonate (PFOS) and perfluorooctanoate (PFOA) concentrations and medical surveillance examinations. J Occup Environ Med 45:260–270
124. Olsen GW, Church TR, Miller JP, Burris JM, Hansen KJ, Lundberg JK, Armitage JB, Herron RM, Medhdizadehkashi Z, Nobiletti JB, O'Neill EM, Mandel JH, Zobel LR (2003) Perfluorooctanesulfonate and other fluorochemicals in the serum of American Red Cross adult blood donors. Environ Health Perspect 111:1892–1901; erratum in: Environ Health Perspect 111:1900
125. Calafat AM, Kuklenyik Z, Caudill SP, Reidy JA, Needham LL (2006) Perfluorochemicals in pooled serum samples from United States residents in 2001 and 2002. Environ Sci Technol 40:2128–2134
126. Kato K, Calafat AM, Wong LY, Wanigatunga AA, Caudill SP, Needham LL (2009) Polyfluoroalkyl compounds in pooled sera from children participating in the National Health and Nutrition Examination Survey 2001-2002. Environ Sci Technol 43:2641–2647
127. Calafat AM, Wong LY, Kuklenyik Z, Reidy JA, Needham LL (2007) Polyfluoroalkyl chemicals in the U.S. population: data from the National Health and Nutrition Examination Survey (NHANES) 2003-2004 and comparisons with NHANES 1999-2000. Environ Health Perspect 115:1596–1602
128. CDC NHANES Report of the 2005-2006 Survey (2010). Online available at http://www.cdc.gov/exposurereport/pdf/Update_Tables.pdf
129. Kärrman A, Mueller JF, van Bavel B, Harden F, Toms LM, Lindström G (2006) Levels of 12 perfluorinated chemicals in pooled Australian serum, collected 2002-2003, in relation to age, gender, and region. Environ Sci Technol 40:3742–3748
130. Harada K, Koizumi A, Saito N, Inoue K, Yoshinaga T, Date C, Fujii S, Hachiya N, Hirosawa I, Koda S, Kusaka Y, Murata K, Omae K, Shimbo S, Takenaka K, Takeshita T,

Todoriki H, Wada Y, Watanabe T, Ikeda M (2007) Historical and geographical aspects of the increasing perfluorooctanoate and perfluorooctane sulfonate contamination in human serum in Japan. Chemosphere 66:293–301
131. Jin Y, Saito N, Harada KH, Inoue K, Koizumi A (2007) Historical trends in human serum levels of perfluorooctanoate and perfluorooctane sulfonate in Shenyang, China. Tohoku J Exp Med 212:63–70
132. Haug LS, Thomsen C, Becher G (2009) Time trends and the influence of age and gender on serum concentrations of perfluorinated compounds in archived human samples. Environ Sci Technol 43:2131–2136
133. Toms LM, Calafat AM, Kato K, Thompson J, Harden F, Hobson P, Sjödin A, Mueller JF (2009) Polyfluoroalkyl chemicals in pooled blood serum from infants, children, and adults in Australia. Environ Sci Technol 43:4194–4199
134. Ingelido AM, Marra V, Abballe A, Valentini S, Iacovella N, Barbieri P, Porpora MG, Domenico A, Felip ED (2010) Perfluorooctanesulfonate and perfluorooctanoic acid exposures of the Italian general population. Chemosphere 80:1125–1130
135. Rylander C, Sandanger TM, Frøyland L, Lund E (2010) Dietary patterns and plasma concentrations of perfluorinated compounds in 315 Norwegian women: the NOWAC Postgenome Study. Environ Sci Technol 44:5225–5232
136. Zhang T, Wu Q, Sun HW, Zhang XZ, Yun SH, Kannan K (2010) Perfluorinated compounds in whole blood samples from infants, children, and adults in China. Environ Sci Technol 44:4341–4347
137. Olsen GW, Mair DC, Church TR, Ellefson ME, Reagen WK, Boyd TM, Herron RM, Medhdizadehkashi Z, Nobiletti JB, Rios JA, Butenhoff JL, Zobel LR (2008) Decline in perfluorooctanesulfonate and other polyfluoroalkyl chemicals in American Red Cross adult blood donors, 2000-2006. Environ Sci Technol 42:4989–4995
138. Inoue K, Okada F, Ito R, Kato S, Sasaki S, Nakajima S, Uno A, Saijo Y, Sata F, Yoshimura Y, Kishi R, Nakazawa H (2004) Perfluorooctane sulfonate (PFOS) and related perfluorinated compounds in human maternal and cord blood samples: assessment of PFOS exposure in a susceptible population during pregnancy. Environ Health Perspect 112: 1204–1207
139. Apelberg BJ, Witter FR, Herbstman JB, Calafat AM, Halden RU, Needham LL, Goldman LR (2007) Cord serum concentrations of perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) in relation to weight and size at birth. Environ Health Perspect 115:1670–1676
140. Monroy R, Morrison K, Teo K, Atkinson S, Kubwabo C, Stewart B, Foster WG (2008) Serum levels of perfluoroalkyl compounds in human maternal and umbilical cord blood samples. Environ Res 108:56–62
141. So MK, Yamashita N, Taniyasu S, Jiang Q, Giesy JP, Chen K, Lam PK (2006) Health risks in infants associated with exposure to perfluorinated compounds in human breast milk from Zhoushan, China. Environ Sci Technol 40:2924–2929
142. Tao L, Ma J, Kunisue T, Libelo EL, Tanabe S, Kannan K (2008) Perfluorinated compounds in human breast milk from several Asian countries, and in infant formula and dairy milk from the United States. Environ Sci Technol 42:8597–8602
143. von Ehrenstein OS, Fenton SE, Kato K, Kuklenyik Z, Calafat AM, Hines EP (2009) Polyfluoroalkyl chemicals in the serum and milk of breastfeeding women. Reprod Toxicol 27:239–245
144. Sundström M, Ehresman DJ, Bignert A, Butenhoff JL, Olsen GW, Chang SC, Bergman A (2011) A temporal trend study (1972-2008) of perfluorooctanesulfonate, perfluorohexanesulfonate, and perfluorooctanoate in pooled human milk samples from Stockholm, Sweden. Environ Int 37:178–183
145. Liu J, Li J, Zhao Y, Wang Y, Zhang L, Wu Y (2010) The occurrence of perfluorinated alkyl compounds in human milk from different regions of China. Environ Int 36:433–438
146. Fromme H, Mosch C, Morovitz M, Alba-Alejandre I, Boehmer S, Kiranoglu M, Faber F, Hannibal I, Genzel-Boroviczény O, Koletzko B, Völkel W (2010) Pre- and postnatal exposure to perfluorinated compounds (PFCs). Environ Sci Technol 44:7123–7129

147. Kim SK, Lee KT, Kang CS, Tao L, Kannan K, Kim KR, Kim CK, Lee JS, Park PS, Yoo YW, Ha JY, Shin YS, Lee JH (2011) Distribution of perfluorochemicals between sera and milk from the same mothers and implications for prenatal and postnatal exposures. Environ Pollut 159:169–174
148. Begley TH, White K, Honigfort P, Twaroski ML, Neches R, Walker RA (2005) Perfluorochemicals: potential sources of and migration from food packaging. Food Addit Contam 22:1023–1031
149. Tittlemier SA, Pepper K, Seymour C, Moisey J, Bronson R, Cao XL, Dabeka RW (2007) Dietary exposure of Canadians to perfluorinated carboxylates and perfluorooctane sulfonate via consumption of meat, fish, fast foods, and food items prepared in their packaging. J Agric Food Chem 55:3203–3210
150. Kärrman A, Harada KH, Inoue K, Takasuga T, Ohi E, Koizumi A (2009) Relationship between dietary exposure and serum perfluorochemical (PFC) levels—a case study. Environ Int 35:712–717
151. Haug LS, Thomsen C, Brantsaeter AL, Kvalem HE, Haugen M, Becher G, Alexander J, Meltzer HM, Knutsen HK (2010) Diet and particularly seafood are major sources of perfluorinated compounds in humans. Environ Int 36:772–778
152. Haug LS, Salihovic S, Jogsten IE, Thomsen C, van Bavel B, Lindström G, Becher G (2010) Levels in food and beverages and daily intake of perfluorinated compounds in Norway. Chemosphere 80:1137–1143
153. Vestergren R, Cousins IT (2009) Tracking the pathways of human exposure to perfluorocarboxylates. Environ Sci Technol 43:5565–5575
154. European Food Safety Authority (2008) Opinion of the Scientific Panel on Contaminants in the Food chain on Perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and their salts. The EFSA Journal 653:1–131
155. Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (2006) COT statement on the tolerable daily intake for perfluorooctanoic acid
156. Committee on Toxicity of Chemicals in Food, Consumer Products and The Environment (2006) COT statement on the tolerable daily intake for perfluorooctane sulfonate
157. German Federal Institute for Risk Assessment (2006) High levels of perfluorinated organic surfactants in fish are likely to be harmful to human health Statement No. 21/2006
158. Bartell SM, Calafat AM, Lyu C, Kato K, Ryan PB, Steenland K (2010) Rate of decline in serum PFOA concentrations after granular activated carbon filtration at two public water systems in Ohio and West Virginia. Environ Health Perspect 118:222–228
159. Johnson JD, Gibson SJ, Ober RE (1984) Cholestyramine-enhanced fecal elimination of carbon-14 in rats after administration of ammonium [^{14}C]perfluorooctanoate or potassium [^{14}C]perfluorooctanesulfonate. Fundam Appl Toxicol 4:972–976
160. Hanhijarvi H, Ylinen M, Haaranen T, Nevalainen T (1988) A proposed species difference in the renal excretion of perfluorooctanoic acid in the beagle dog and rat. In: Beynen AC, Solleveld HA (eds) New development in biosciences: their implications for laboratory animal sciences. Martinus Nijhoff Publishers, Dordrecht, The Netherlands, pp 409–412
161. Vanden Heuvel JP, Kuslikis BI, Van Ragelghem ML, Peterson RE (1991) Tissue distribution, metabolism, and elimination of perfluorooctanoic acid in male and female rats. J Biochem Toxicol 6:83–92
162. Kudo N, Katakura M, Sato Y, Kawashima Y (2002) Sex hormone-regulated renal transport of perfluorooctanoic acid. Chem Biol Interact 139:301–316
163. Ohmori K, Kudo N, Katayama K, Kawashima Y (2003) Comparison of the toxicokinetics between perfluorocarboxylic acids with different carbon chain length. Toxicology 184: 135–140
164. Butenhoff JL, Kennedy GL Jr, Hinderliter PM, Lieder PH, Jung R, Hansen KJ, Gorman GS, Noker PE, Thomford PJ (2004) Pharmacokinetics of perfluorooctanoate in cynomolgus monkeys. Toxicol Sci 82:394–406

165. Harada K, Inoue K, Morikawa A, Yoshinaga T, Saito N, Koizumi A (2005) Renal clearance of perfluorooctane sulfonate and perfluorooctanoate in humans and their species-specific excretion. Environ Res 99:253–261
166. Hinderliter PM, Mylchreest E, Gannon SA, Butenhoff JL, Kennedy GL (2005) Perfluorooctanoate: placental and lactational transport pharmacokinetics in rats. Toxicology 211: 139–148
167. Hundley SG, Sarrif AM, Kennedy GL (2006) Absorption, distribution, and excretion of ammonium perfluorooctanoate (APFO) after oral administration to various species. Drug Chem Toxicol 29:137–145
168. Hinderliter PM, Han X, Kennedy GL, Butenhoff JL (2006) Age effect on perfluorooctanoate (PFOA) plasma concentration in post-weaning rats following oral gavage with ammonium perfluorooctanoate (APFO). Toxicology 225:195–203
169. Olsen GW, Burris JM, Ehresman DJ, Froehlich JW, Seacat AM, Butenhoff JL, Zobel LR (2007) Half-life of serum elimination of perfluorooctanesulfonate, perfluorohexanesulfonate, and perfluorooctanoate in retired fluorochemical production workers. Environ Health Perspect 115:1298–1305
170. Chang SC, Das K, Ehresman DJ, Ellefson ME, Gorman GS, Hart JA, Noker PE, Tan YM, Lieder PH, Lau C, Olsen GW, Butenhoff JL (2008) Comparative pharmacokinetics of perfluorobutyrate in rats, mice, monkeys, and humans and relevance to human exposure via drinking water. Toxicol Sci 104:40–53
171. Lou I, Wambaugh JF, Lau C, Hanson RG, Lindstrom AB, Strynar MJ, Zehr RD, Setzer RW, Barton HA (2009) Modeling single and repeated dose pharmacokinetics of PFOA in mice. Toxicol Sci 107:331–341
172. Olsen GW, Chang SC, Noker PE, Gorman GS, Ehresman DJ, Lieder PH, Butenhoff JL (2009) A comparison of the pharmacokinetics of perfluorobutanesulfonate (PFBS) in rats, monkeys, and humans. Toxicology 256:65–74
173. Chengelis CP, Kirkpatrick JB, Myers NR, Shinohara M, Stetson PL, Sved DW (2009) Comparison of the toxicokinetic behavior of perfluorohexanoic acid (PFHxA) and nonafluorobutane-1-sulfonic acid (PFBS) in cynomolgus monkeys and rats. Reprod Toxicol 27:400–406
174. Benskin JP, De Silva AO, Martin LJ, Arsenault G, McCrindle R, Riddell N, Mabury SA, Martin JW (2009) Disposition of perfluorinated acid isomers in Sprague–Dawley rats; part 1: single dose. Environ Toxicol Chem 28:542–554
175. Tatum-Gibbs K, Wambaugh J, Das KP, Zehr RD, Strynar MJ, Lindstrom AB, Delinsky A, Lau C (2011) Comparative pharmacokinetics of perfluorononanoic acid in rat and mouse. Toxicology 281:48–55
176. Kudo N, Suzuki E, Katakura M, Ohmori K, Noshiro R, Kawashima Y (2001) Comparison of the elimination between perfluorinated fatty acids with different carbon chain length in rats. Chem Biol Interact 134:203–216
177. Buist SC, Cherrington NJ, Klaassen CD (2003) Endocrine regulation of rat organic anion transporters. Drug Metab Dispos 31:559–564
178. Ljubojevic M, Balen D, Breljak D, Kusan M, Anzai N, Bahn A, Burckhardt G, Sabolic I (2007) Renal expression of organic anion transporter OAT2 in rats and mice is regulated by sex hormones. Am J Physiol Renal Physiol 292:F361–372
179. Sundstrom M, Chang SC, Noker PE, Gorman GS, Hart JA, Ehresman DJ, Bergman A, Butenhoff JL (2011) Comparative pharmacokinetics of perfluorohexaneuslfonate (PFHxS) in rats, mice, and monkeys. Repord Toxicol. Epub ahead
180. Buist SC, Klaassen CD (2004) Rat and mouse differences in gender-predominant expression of organic anion transporter (Oat1-3; Slc22a6-8) mRNA levels. Drug Metab Dispos 32: 620–625
181. Yang CH, Glover KP, Han X (2010) Characterization of cellular uptake of perfluorooctanoate via organic anion-transporting polypeptide 1A2, organic anion transporter 4, and urate transporter 1 for their potential roles in mediating human renal reabsorption of perfluorocarboxylates. Toxicol Sci 117:294–302

182. OECD (2002) Hazard assessment of perfluorooctane sulfonate (PFOS) and its salts. Online available at http://www.oecd.org/document/58/0,3343,en_2649_34375_2384378_1_1_1_37465,00.html
183. Fernández Freire P, Pérez Martin JM, Herrero O, Peropadre A, de la Peña E, Hazen MJ (2008) In vitro assessment of the cytotoxic and mutagenic potential of perfluorooctanoic acid. Toxicol In Vitro 22:1228–1233
184. Kim SC, Hong JT, Jang SJ, Kang WS, Yoo HS, Yun YP (1998) Formation of 8-oxodeoxyguanosine in liver DNA and hepatic injury by peroxisome proliferator clofibrate and perfluorodecanoic acid in rats. J Toxicol Sci 23:113–119
185. Loveless SE, Slezak B, Serex T, Lewis J, Mukerji P, O'Connor JC, Donner EM, Frame SR, Korzeniowski SH, Buck RC (2009) Toxicological evaluation of sodium perfluorohexanoate. Toxicology 264:32–44
186. Yao X, Zhong L (2005) Genotoxic risk and oxidative DNA damage in HepG2 cells exposed to perfluorooctanoic acid. Mutat Res 587:38–44
187. Eriksen KT, Raaschou-Nielsen O, Sørensen M, Roursgaard M, Loft S, Møller P (2010) Genotoxic potential of the perfluorinated chemicals PFOA, PFOS, PFBS, PFNA and PFHxA in human HepG2 cells. Mutat Res 700:39–43
188. U.S. Environmental Protection Agency (US EPA) (2005) Draft risk assessment of the potential human health effects associated with exposure to perfluorooctanoic acid and its salts. Online available at http://www.epa.gov/opptintr/pfoa/pubs/pfoarisk.html
189. Hardisty JF, Willson GA, Brown WR, McConnell EE, Frame SR, Gaylor DW, Kennedy GL, Butenhoff JL (2010) Pathology Working Group review and evaluation of proliferative lesions of mammary gland tissues in female rats fed ammonium perfluorooctanoate (APFO) in the diet for 2 years. Drug Chem Toxicol 33:131–137
190. Liu RC, Hurtt ME, Cook JC, Biegel LB (1996) Effect of the peroxisome proliferator, ammonium perfluorooctanoate (C8), on hepatic aromatase activity in adult male Crl:CD BR (CD) rats. Fundam Appl Toxicol 30:220–228
191. Biegel LB, Hurtt ME, Frame SR, O'Connor JC, Cook JC (2001) Mechanisms of extrahepatic tumor induction by peroxisome proliferators in male CD rats. Toxicol Sci 60:44–55
192. Tilton SC, Orner GA, Benninghoff AD, Carpenter HM, Hendricks JD, Pereira CB, Williams DE (2008) Genomic profiling reveals an alternate mechanism for hepatic tumor promotion by perfluorooctanoic acid in rainbow trout. Environ Health Perspect 116:1047–1055
193. van Rafelghem MJ, Mattie DR, Bruner RH, Andersen ME (1987) Pathological and hepatic ultrastructural effects of a single dose of perfluoro-n-decanoic acid in the rat, hamster, mouse, and guinea pig. Fundam Appl Toxicol 9:522–540
194. Kudo N, Suzuki-Nakajima E, Mitsumoto A, Kawashima Y (2006) Responses of the liver to perfluorinated fatty acids with different carbon chain length in male and female mice:in relation to induction of hepatomegaly, peroxisomal beta-oxidation and microsomal 1-acylglycerophosphocholine acyltransferase. Biol Pharm Bull 29:1952–1957
195. Ehresman DJ, Chang S, Bjork JA, Hart JA, Lieder PH, Wallace KB, Butenhoff JL (2007) Increased acyl CoA oxidase activity in rats after five consecutive daily doses of perfluorobutanesulfonate, perfluorohexanesulfonate, and perfluorooctanesulfonate. Toxicologist 96:179
196. Lieder PH, York RG, Hakes DC, Chang SC, Butenhoff JL (2009) A two-generation oral gavage reproduction study with potassium perfluorobutanesulfonate (K^+PFBS) in Sprague Dawley rats. Toxicology 259:33–45
197. Foreman JE, Chang SC, Ehresman DJ, Butenhoff JL, Anderson CR, Palkar PS, Kang BH, Gonzalez FJ, Peters JM (2009) Differential hepatic effects of perfluorobutyrate mediated by mouse and human PPAR-α. Toxicol Sci 110:204–211
198. Chengelis CP, Kirkpatrick JB, Radovsky A, Shinohara M (2009) A 90-day repeated dose oral (gavage) toxicity study of perfluorohexanoic acid (PFHxA) in rats (with functional observational battery and motor activity determinations). Reprod Toxicol 27:342–351

199. Kudo N, Bandai N, Suzuki E, Katakura M, Kawashima Y (2000) Induction by perfluorinated fatty acids with different carbon chain length of peroxisomal beta-oxidation in the liver of rats. Chem Biol Interact 124:119–132
200. Hoff PT, Scheirs J, Van de Vijver K, Van Dongen W, Esmans EL, Blust R, De Coen W (2004) Biochemical effect evaluation of perfluorooctane sulfonic acid-contaminated wood mice (*Apodemus sylvaticus*). Environ Health Perspect 112:681–686
201. Curran I, Hierlihy SL, Liston V, Pantazopoulos P, Nunnikhoven A, Tittlemier S, Barker M, Trick K, Bondy G (2008) Altered fatty acid homeostasis and related toxicologic sequelae in rats exposed to dietary potassium perfluorooctanesulfonate (PFOS). J Toxicol Environ Health A 71:1526–1541
202. Kudo N, Mizuguchi H, Yamamoto A, Kawashima Y (1999) Alterations by perfluorooctanoic acid of glycerolipid metabolism in rat liver. Chem Biol Interact 118:69–83
203. Chang SC, Noker PE, Gorman GS, Gibson SJ, Hart JA, Ehresman DJ, Butenhoff JL (2011) Comparative pharmacokinetics of perfluorooctanesulfonate (PFOS) in rats, mice, and monkeys. Reprod Toxicol. Epub ahead
204. Guruge KS, Yeung LW, Yamanaka N, Miyazaki S, Lam PK, Giesy JP, Jones PD, Yamashita N (2006) Gene expression profiles in rat liver treated with perfluorooctanoic acid (PFOA). Toxicol Sci 89:93–107
205. Martin MT, Breman R, Hu W, Ayanoglu E, Lau C, Ren H, Wood CR, Corton JC, Kavlock RJ, Dix DJ (2007) Toxicogenomic study of triazole fungicides and perfluoroalkyl acids in rat livers accurately categorizes chemicals and identifies mechanisms of toxicity. Toxicol Sci 97:595–613
206. Rosen MB, Thibodeaux JR, Wood CR, Zehr RD, Schmid JE, Lau C (2007) Gene expression profiling in the lung and liver of PFOA-exposed mouse fetuses. Toxicology 239:15–33
207. Shipley JM, Hurst CH, Tanaka SS, DeRoos FL, Butenhoff JL, Seacat AM, Waxman DJ (2004) trans-Activation of PPARα and induction of PPARα target genes by perfluorooctane-based chemicals. Toxicol Sci 80:151–160
208. Kato K, Wong LY, Jia LT, Kuklenyik Z, Calafat AM (2011) Trends in exposure to polyfluoroalkyl chemicals in the U.S. population: 1999–2008. Environ Sci Technol 45: 8037–8045
209. Wolf DC, Moore T, Abbott BD, Rosen MB, Das KP, Zehr RD, Lindstrom AB, Strynar MJ, Lau C (2008) Comparative hepatic effects of perfluorooctanoic acid and WY 14,643 in PPARα-knocked out and wild-type mice. Toxicol Pathol 36:632–639
210. Rosen MB, Schmid JR, Zehr RD, Das KP, Abbott BD, Lau C (2010) Gene expression profiling in wild-type and PPARα-null mice exposed to perfluorooctane sulfonate reveals PPARα-independent effects. PPAR Res. Epub ahead
211. Starkov AA, Wallace KB (2002) Structural determinants of fluorochemical-induced mitochondrial dysfunction. Toxicol Sci 66:244–252
212. Elcombe CR, Elcombe BM, Foster JR, Farrar DG, Jung R, Chang SC, Kennedy GL, Butenhoff JL (2010) Hepatocellular hypertrophy and cell proliferation in Sprague–Dawley rats following dietary exposure to ammonium perfluorooctanoate occurs through increased activation of the xenosensor nuclear receptors PPARα and CAR/PXR. Arch Toxicol 84:787–798
213. Thibodeaux JR, Hanson RG, Rogers JM, Grey BE, Barbee BD, Richards JH, Butenhoff JL, Stevenson LA, Lau C (2003) Exposure to perfluorooctane sulfonate during pregnancy in rat and mouse. I. Maternal and prenatal evaluations. Toxicol Sci 74:369–381
214. Luebker DJ, Case MT, York RG, Moore JA, Hansen KJ, Butenhoff JL (2005) Two-generation reproduction and cross-foster studies of perfluorooctanesulfonate (PFOS) in rats. Toxicology 215:126–148
215. Lau C, Thibodeaux JR, Hanson RG, Narotsky MG, Rogers JM, Lindstrom AB, Strynar MJ (2006) Effects of perfluorooctanoic acid exposure during pregnancy in the mouse. Toxicol Sci 90:510–518
216. Era S, Harada KH, Toyoshima M, Inoue K, Minata M, Saito N, Takigawa T, Shiota K, Koizumi A (2009) Cleft palate caused by perfluorooctane sulfonate is caused mainly by extrinsic factors. Toxicology 256:42–47

217. Das KP, Grey BE, Zehr RD, Wood CR, Butenhoff JL, Chang SC, Ehresman DJ, Tan YM, Lau C (2008) Effects of perfluorobutyrate exposure during pregnancy in the mouse. Toxicol Sci 105:173–181
218. Harris MW, Birnbaum LS (1989) Developmental toxicity of perfluorodecanoic acid in C57BL/6N mice. Fundam Appl Toxicol 12:442–448
219. Lau C, Thibodeaux JR, Hanson RG, Rogers JM, Grey BE, Stanton ME, Butenhoff JL, Stevenson LA (2003) Exposure to perfluorooctane sulfonate during pregnancy in rat and mouse. II. Postnatal evaluation. Toxicol Sci 74:382–392
220. Luebker DJ, York RG, Hansen KJ, Moore JA, Butenhoff JL (2005) Neonatal mortality from *in utero* exposure to perfluorooctanesulfonate (PFOS) in Sprague–Dawley rats: dose-response, and biochemical and pharmacokinetic parameters. Toxicology 215:149–169
221. Yu WG, Liu W, Jin YH, Liu XH, Wang FQ, Liu L, Nakayama SF (2009) Prenatal and postnatal impact of perfluorooctane sulfonate (PFOS) on rat development: a cross-foster study on chemical burden and thyroid hormone system. Environ Sci Technol 43:8416–8422
222. Grasty RC, Wolf DC, Grey BE, Lau CS, Rogers JM (2003) Prenatal window of susceptibility to perfluorooctane sulfonate-induced neonatal mortality in the Sprague–Dawley rat. Birth Defects Res B Dev Reprod Toxicol 68:465–471
223. Grasty RC, Bjork JA, Wallace KB, Wolf DC, Lau CS, Rogers JM (2005) Effects of prenatal perfluorooctane sulfonate (PFOS) exposure on lung maturation in the perinatal rat. Birth Defects Res B Dev Reprod Toxicol 74:405–416
224. Borg D, Bogdanska J, Sundström M, Nobel S, Håkansson H, Bergman A, Depierre JW, Halldin K, Bergström U (2010) Tissue distribution of (35)S-labelled perfluorooctane sulfonate (PFOS) in C57Bl/6 mice following late gestational exposure. Reprod Toxicol 30: 558–565
225. Butenhoff JL, Kennedy GL, Frame SR, O'Connor JC, York RG (2004) The reproductive toxicology of ammonium perfluorooctanoate (APFO) in the rat. Toxicology 196:95–116
226. Wolf CJ, Fenton SE, Schmid JE, Calafat AM, Kuklenyik Z, Bryant XA, Thibodeaux J, Das KP, White SS, Lau CS, Abbott BD (2007) Developmental toxicity of perfluorooctanoic acid in the CD-1 mouse after cross-foster and restricted gestational exposures. Toxicol Sci 95:462–473
227. White SS, Calafat AM, Kuklenyik Z, Villanueva L, Zehr RD, Helfant L, Strynar MJ, Lindstrom AB, Thibodeaux JR, Wood C, Fenton SE (2007) Gestational PFOA exposure of mice is associated with altered mammary gland development in dams and female offspring. Toxicol Sci 96:133–144
228. Hines EP, White SS, Stanko JP, Gibbs-Flournoy EA, Lau C, Fenton SE (2009) Phenotypic dichotomy following developmental exposure to perfluorooctanoic acid (PFOA) in female CD-1 mice: low doses induce elevated serum leptin and insulin, and overweight in mid-life. Mol Cell Endocrinol 304:97–105
229. Barker DJ (2007) Obesity and early life. Obes Rev 8(Suppl 1):45–49
230. White SS, Kato K, Jia LT, Basden BJ, Calafat AM, Hines EP, Stanko JP, Wolf CJ, Abbott BD, Fenton SE (2009) Effects of perfluorooctanoic acid on mouse mammary gland development and differentiation resulting from cross-foster and restricted gestational exposures. Reprod Toxicol 27:289–298
231. Abbott BD (2009) Review of the expression of peroxisome proliferator-activated receptors alpha (PPARα), β (PPARβ), and γ (PPARγ) in rodent and human development. Reprod Toxicol 27:246–257
232. Abbott BD, Wolf CJ, Schmid JE, Das K, Zehr RD, Helfant L, Nakayama S, Lindstrom AB, Strynar MJ, Lau C (2007) Perfluorooctanoic acid (PFOA)-induced developmental toxicity in the mouse is dependent on expression of peroxisome proliferator activated receptor-alpha (PPARα). Toxicol Sci 98:571–581
233. Abbott BD, Wolf CJ, Das KP, Zehr RD, Schmid JE, Lindstrom AB, Strynar MJ, Lau C (2009) Developmental toxicity of perfluorooctane sulfonate (PFOS) is not dependent on

expression of peroxisome proliferator activated receptor-α (PPARα) in the mouse. Reprod Toxicol 27:258–265
234. Wolf CJ, Zehr RD, Schmid JE, Lau C, Abbott BD (2010) Developmental effects of perfluorononanoic acid in the mouse are dependent on peroxisome proliferator-activated receptor-α. PPAR Res. Epub ahead
235. Butenhoff JL, Chang SC, Ehresman DJ, York RG (2009) Evaluation of potential reproductive and developmental toxicity of potassium perfluorohexanesulfonate in Sprague Dawley rats. Reprod Toxicol 27:331–341
236. Yang Q, Xie Y, Depierre JW (2000) Effects of peroxisome proliferators on the thymus and spleen of mice. Clin Exp Immunol 122:219–226
237. Yang Q, Xie Y, Eriksson AM, Nelson BD, DePierre JW (2001) Further evidence for the involvement of inhibition of cell proliferation and development in thymic and splenic atrophy induced by the peroxisome proliferator perfluoroctanoic acid in mice. Biochem Pharmacol 62:1133–1140
238. Yang Q, Abedi-Valugerdi M, Xie Y, Zhao XY, Möller G, Nelson BD, DePierre JW (2002) Potent suppression of the adaptive immune response in mice upon dietary exposure to the potent peroxisome proliferator, perfluorooctanoic acid. Int Immunopharmacol 2:389–397
239. Yang Q, Xie Y, Alexson SE, Nelson BD, DePierre JW (2002) Involvement of the peroxisome proliferator-activated receptor α in the immunomodulation caused by peroxisome proliferators in mice. Biochem Pharmacol 63:1893–1900
240. DeWitt JC, Shnyra A, Badr MZ, Loveless SE, Hoban D, Frame SR, Cunard R, Anderson SE, Meade BJ, Peden-Adams MM, Luebke RW, Luster MI (2009) Immunotoxicity of perfluorooctanoic acid and perfluorooctane sulfonate and the role of peroxisome proliferator-activated receptor α. Crit Rev Toxicol 39:76–94
241. Fairley KJ, Purdy R, Kearns S, Anderson SE, Meade BJ (2007) Exposure to the immunosuppressant, perfluorooctanoic acid, enhances the murine IgE and airway hyperreactivity response to ovalbumin. Toxicol Sci 97:375–383
242. Son HY, Lee S, Tak EN, Cho HS, Shin HI, Kim SH, Yang JH (2009) Perfluorooctanoic acid alters T lymphocyte phenotypes and cytokine expression in mice. Environ Toxicol 24: 580–588
243. Dewitt JC, Copeland CB, Strynar MJ, Luebke RW (2008) Perfluorooctanoic acid-induced immunomodulation in adult C57BL/6J or C57BL/6N female mice. Environ Health Perspect 116:644–650
244. Loveless SE, Hoban D, Sykes G, Frame SR, Everds NE (2008) Evaluation of the immune system in rats and mice administered linear ammonium perfluorooctanoate. Toxicol Sci 105:86–96
245. DeWitt JC, Copeland CB, Luebke RW (2009) Suppression of humoral immunity by perfluorooctanoic acid is independent of elevated serum corticosterone concentration in mice. Toxicol Sci 109:106–112
246. Fang X, Zhang L, Feng Y, Zhao Y, Dai J (2008) Immunotoxic effects of perfluorononanoic acid on BALB/c mice. Toxicol Sci 105:312–321
247. Fang X, Feng Y, Shi Z, Dai J (2009) Alterations of cytokines and MAPK signaling pathways are related to the immunotoxic effect of perfluorononanoic acid. Toxicol Sci 108:367–376
248. Fang X, Feng Y, Wang J, Dai J (2010) Perfluorononanoic acid-induced apoptosis in rat spleen involves oxidative stress and the activation of caspase-independent death pathway. Toxicology 267:54–59
249. Peden-Adams MM, EuDaly JG, Dabra S, EuDaly A, Heesemann L, Smythe J, Keil DE (2007) Suppression of humoral immunity following exposure to the perfluorinated insecticide sulfluramid. J Toxicol Environ Health A 70:1130–1141
250. Qazi MR, Xia Z, Bogdanska J, Chang SC, Ehresman DJ, Butenhoff JL, Nelson BD, DePierre JW, Abedi-Valugerdi M (2009) The atrophy and changes in the cellular compositions of the thymus and spleen observed in mice subjected to short-term exposure to perfluorooctanesulfonate are high-dose phenomena mediated in part by peroxisome proliferator-activated receptor-α (PPARα). Toxicology 260:68–76

251. Lefebvre DE, Curran I, Armstrong C, Coady L, Parenteau M, Liston V, Barker M, Aziz S, Rutherford K, Bellon-Gagnon P, Shenton J, Mehta R, Bondy G (2008) Immunomodulatory effects of dietary potassium perfluorooctane sulfonate (PFOS) exposure in adult Sprague–-Dawley rats. J Toxicol Environ Health A 71:1516–1525
252. Peden-Adams MM, Keller JM, Eudaly JG, Berger J, Gilkeson GS, Keil DE (2008) Suppression of humoral immunity in mice following exposure to perfluorooctane sulfonate. Toxicol Sci 104:144–154
253. Zheng L, Dong GH, Jin YH, He QC (2009) Immunotoxic changes associated with a 7-day oral exposure to perfluorooctanesulfonate (PFOS) in adult male C57BL/6 mice. Arch Toxicol 83:679–689
254. Dong GH, Zhang YH, Zheng L, Liu W, Jin YH, He QC (2009) Chronic effects of perfluorooctanesulfonate exposure on immunotoxicity in adult male C57BL/6 mice. Arch Toxicol 83:805–815
255. Dong, G.-H., Zhang, Y.-H., Zheng, L., Liang, Z.-F., Jin, Y.-H, and He, Q.-C. (2012), Subchronic effects of perfluorooctanesulfonate exposure on inflammation in adult male C57BL/6 mice. Environ Toxicol 27:285–296
256. Qazi MR, Nelson BD, Depierre JW, Abedi-Valugerdi M (2010) 28-Day dietary exposure of mice to a low total dose (7 mg/kg) of perfluorooctanesulfonate (PFOS) alters neither the cellular compositions of the thymus and spleen nor humoral immune responses: does the route of administration play a pivotal role in PFOS-induced immunotoxicity? Toxicology 267:132–139
257. Keil DE, Mehlmann T, Butterworth L, Peden-Adams MM (2008) Gestational exposure to perfluorooctane sulfonate suppresses immune function in B6C3F1 mice. Toxicol Sci 103: 77–85
258. Guruge KS, Hikono H, Shimada N, Murakami K, Hasegawa J, Yeung LW, Yamanaka N, Yamashita N (2009) Effect of perfluorooctane sulfonate (PFOS) on influenza A virus-induced mortality in female B6C3F1 mice. J Toxicol Sci 34:687–691
259. Qazi MR, Bogdanska J, Butenhoff JL, Nelson BD, DePierre JW, Abedi-Valugerdi M (2009) High-dose, short-term exposure of mice to perfluorooctanesulfonate (PFOS) or perfluorooctanoate (PFOA) affects the number of circulating neutrophils differently, but enhances the inflammatory responses of macrophages to lipopolysaccharide (LPS) in a similar fashion. Toxicology 262:207–214
260. Qazi MR, Abedi MR, Nelson BD, DePierre JW, Abedi-Valugerdi M (2010) Dietary exposure to perfluorooctanoate or perfluorooctane sulfonate induces hypertrophy in centrilobular hepatocytes and alters the hepatic immune status in mice. Int Immunopharmacol 10: 1420–1427
261. Jensen AA, Leffers H (2008) Emerging endocrine disrupters: perfluoroalkylated substances. Int J Androl 31:161–169
262. Langley AE, Pilcher GD (1985) Thyroid, bradycardic and hypothermic effects of perfluoro-n-decanoic acid in rats. J Toxicol Environ Health 15:485–491
263. Gutshall DM, Pilcher GD, Langley AE (1989) Mechanism of the serum thyroid hormone lowering effect of perfluoro-n-decanoic acid (PFDA) in rats. J Toxicol Environ Health 28: 53–65
264. Seacat AM, Thomford PJ, Hansen KJ, Olsen GW, Case MT, Butenhoff JL (2002) Subchronic toxicity studies on perfluorooctanesulfonate potassium salt in *Cynomolgus* monkeys. Toxicol Sci 68:249–264
265. Yu WG, Liu W, Jin YH (2009) Effects of perfluorooctane sulfonate on rat thyroid hormone biosynthesis and metabolism. Environ Toxicol Chem 28:990–996
266. Chang SC, Ehresman DJ, Bjork JA, Wallace KB, Parker GA, Stump DG, Butenhoff JL (2009) Gestational and lactational exposure to potassium perfluorooctanesulfonate (K^+PFOS) in rat: toxicokinetics, thyroid hormone status, and related gene expression. Reprod Toxicol 27:387–399

267. Chang S, Thibodeaux JR, Eastvold ML, Ehresman DJ, Bjork J, Froehlich JW, Lau C, Singh RJ, Wallace KB, Butenhoff JL (2007) Negative bias from analog methods used in the analysis of free thyroid hormones in rat serum containing perfluorooctanesulfonate (PFOS). Toxicology 234:21–33
268. Chang S, Thibodeaux JR, Eastvold ML, Ehresman DJ, Bjork JA, Froehlich JW, Lau C, Singh RJ, Wallace KB, Butenhoff JL (2008) Thyroid hormone status and pituitary function in adult rats given oral doses of perfluorooctanesulfonate (PFOS). Toxicology 243:330–339
269. Weiss JM, Andersson PL, Lamoree MH, Leonards PEG, van Leeuwen SPJ, Hamers T (2009) Competitive binding of poly- and perfluorinated compounds to the thyroid hormone transport protein transthyretin. Toxicol Sci 109:206–216
270. Bjork JA, Lau C, Chang S, Butenhoff JL, Wallace KB (2008) Perfluorooctane sulfonate-induced changes in neonatal rat liver gene expression. Toxicology 251:8–20
271. Cook JC, Murray SM, Frame SR, Hurtt ME (1992) Induction of Leydig cell adenomas by ammonium perfluorooctanoate: a possible endocrine-related mechanism. Toxicol Appl Pharmacol 113:209–217
272. Liu C, Du Y, Zhou B (2007) Evaluation of estrogenic activities and mechanism of action of perfluorinated chemicals determined by vitellogenin induction in primary cultured tilapia hepatocytes. Aquat Toxicol 85:267–277
273. Ishibashi H, Ishida H, Matsuoka M, Tominaga N, Arizono K (2007) Estrogenic effects of fluorotelomer alcohols for human estrogen receptor isoforms α and β *in vitro*. Biol Pharm Bull 30:1358–1359
274. Ishibashi H, Yamauchi R, Matsuoka M, Kim JW, Hirano M, Yamaguchi A, Tominaga N, Arizono K (2008) Fluorotelomer alcohols induce hepatic vitellogenin through activation of the estrogen receptor in male medaka (*Oryzias latipes*). Chemosphere 71:1853–1859
275. Zhao B, Chu Y, Hardy DO, Li XK, Ge RS (2010) Inhibition of 3β- and 17β-hydroxysteroid dehydrogenase activities in rat Leydig cells by perfluorooctane acid. J Steroid Biochem Mol Biol 118:13–17
276. Liao C, Wang T, Cui L, Zhou Q, Duan S, Jiang G (2009) Changes in synaptic transmission, calcium current, and neurite growth by perfluorinated compounds are dependent on the chain length and functional group. Environ Sci Technol 43:2099–2104
277. Shi Z, Zhang H, Liu Y, Xu M, Dai J (2007) Alterations in gene expression and testosterone synthesis in the testes of male rats exposed to perfluorododecanoic acid. Toxicol Sci 98: 206–215
278. Shi Z, Ding L, Zhang H, Feng Y, Xu M, Dai J (2009) Chronic exposure to perfluorododecanoic acid disrupts testicular steroidogenesis and the expression of related genes in male rats. Toxicol Lett 188:192–200
279. Shi Z, Zhang H, Ding L, Feng Y, Xu M, Dai J (2009) The effect of perfluorododecanonic acid on endocrine status, sex hormones and expression of steroidogenic genes in pubertal female rats. Reprod Toxicol 27:352–359
280. Shi Z, Zhang H, Ding L, Feng Y, Wang J, Dai J (2010) Proteomic analysis for testis of rats chronically exposed to perfluorododecanoic acid. Toxicol Lett 192:179–188
281. Shi Z, Feng Y, Wang J, Zhang H, Ding L, Dai J (2010) Perfluorododecanoic acid-induced steroidogenic inhibition is associated with steroidogenic acute regulatory protein and reactive oxygen species in cAMP-stimulated Leydig cells. Toxicol Sci 114:285–294
282. Slotkin TA, MacKillop EA, Melnick RL, Thayer KA, Seidler FJ (2008) Developmental neurotoxicity of perfluorinated chemicals modeled *in vitro*. Environ Health Perspect 116: 716–722
283. Liao CY, Li XY, Wu B, Duan S, Jiang GB (2008) Acute enhancement of synaptic transmission and chronic inhibition of synaptogenesis induced by perfluorooctane sulfonate through mediation of voltage-dependent calcium channel. Environ Sci Technol 42:5335–5341
284. Fuentes S, Vicens P, Colomina MT, Domingo JL (2007) Behavioral effects in adult mice exposed to perfluorooctane sulfonate (PFOS). Toxicology 242:123–129

285. Liu X, Liu W, Jin Y, Yu W, Liu L, Yu H (2010) Effects of subchronic perfluorooctane sulfonate exposure of rats on calcium-dependent signaling molecules in the brain tissue. Arch Toxicol 84:471–479
286. Sato I, Kawamoto K, Nishikawa Y, Tsuda S, Yoshida M, Yaegashi K, Saito N, Liu W, Jin Y (2009) Neurotoxicity of perfluorooctane sulfonate (PFOS) in rats and mice after single oral exposure. J Toxicol Sci 34:569–574
287. Butenhoff JL, Ehresman DJ, Chang SC, Parker GA, Stump DG (2009) Gestational and lactational exposure to potassium perfluorooctanesulfonate (K^+PFOS) in rats: developmental neurotoxicity. Reprod Toxicol 27:319–330
288. Liu X, Liu W, Jin Y, Yu W, Wang F, Liu L (2010) Effect of gestational and lactational exposure to perfluorooctanesulfonate on calcium-dependent signaling molecules gene expression in rats' hippocampus. Arch Toxicol 84:71–79
289. Wang F, Liu W, Jin Y, Dai J, Yu W, Liu X, Liu L (2010) Transcriptional effects of prenatal and neonatal exposure to PFOS in developing rat brain. Environ Sci Technol 44:1847–1853
290. Johansson N, Fredriksson A, Eriksson P (2008) Neonatal exposure to perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) causes neurobehavioural defects in adult mice. Neurotoxicology 29:160–169
291. Johansson N, Eriksson P, Viberg H (2009) Neonatal exposure to PFOS and PFOA in mice results in changes in proteins which are important for neuronal growth and synaptogenesis in the developing brain. Toxicol Sci 108:412–418
292. Pinkas A, Slotkin TA, Brick-Turin Y, Van der Zee EA, Yanai J (2010) Neurobehavioral teratogenicity of perfluorinated alkyls in an avian model. Neurotoxicol Teratol 32:182–186
293. Wolf CJ, Takacs MR, Schmid JE, Lau C, Abbott BD (2008) Differential activation of mouse and human peroxisome proliferator-activated receptor-α (PPARα) by perfluoroalkyl carboxylates and sulfonates of different chain lengths. Toxicol Sci 106:162–171
294. Klaunig JE, Babich MA, Baetcke KP, Cook JC, Corton JC, David RM, DeLuca JG, Lai DY, McKee RH, Peters JM, Roberts RA, Fenner-Crisp PA (2003) PPARα agonist-induced rodent tumors: modes of action and human relevance. Crit Rev Toxicol 33:655–780
295. Cohen SM, Meek ME, Klaunig JE, Patton DE, Fenner-Crisp PA (2003) The human relevance of information on carcinogenic modes of action: overview. Crit Rev Toxicol 33: 581–589
296. Nakamura T, Ito Y, Yanagiba Y, Ramdhan DH, Kono Y, Naito H, Hayashi Y, Li Y, Aoyama T, Gonzalez FJ, Nakajima T (2009) Microgram-order ammonium perfluorooctanoate may activate mouse peroxisome proliferator-activated receptor alpha, but not human PPARα. Toxicology 265:27–33
297. Guyton KZ, Chiu WA, Bateson TF, Jinot J, Scott CS, Brown RC, Caldwell JC (2009) A reexamination of the PPAR-α activation mode of action as a basis for assessing human cancer risks of environmental contaminants. Environ Health Perspect 117:1664–1672
298. Zhang H, Shi Z, Liu Y, Wei Y, Dai J (2008) Lipid homeostasis and oxidative stress in the liver of male rats exposed to perfluorododecanoic acid. Toxicol Appl Pharmacol 227:16–25
299. Qian Y, Ducatman A, Ward R, Leonard S, Bukowski V, Lan Guo N, Shi X, Vallyathan V, Castranova V (2010) Perfluorooctane sulfonate (PFOS) induces reactive oxygen species (ROS) production in human microvascular endothelial cells: role in endothelial permeability. J Toxicol Environ Health A 73:819–836
300. Xie W, Wu Q, Kania-Korwel I, Tharappel JC, Telu S, Coleman MC, Glauert HP, Kannan K, Mariappan SV, Spitz DR, Weydert J, Lehmler HJ (2009) Subacute exposure to N-ethyl perfluorooctanesulfonamidoethanol results in the formation of perfluorooctanesulfonate and alters superoxide dismutase activity in female rats. Arch Toxicol 83:909–924
301. Liu C, Zhang X, Chang H, Jones P, Wiseman S, Naile J, Hecker M, Giesy JP, Zhou B (2010) Effects of fluorotelomer alcohol 8:2 FTOH on steroidogenesis in H295R cells: targeting the cAMP signalling cascade. Toxicol Appl Pharmacol 247:222–228
302. Feng Y, Shi Z, Fang X, Xu M, Dai J (2009) Perfluorononanoic acid induces apoptosis involving the Fas death receptor signaling pathway in rat testis. Toxicol Lett 190:224–230

303. Guerrero-Preston R, Goldman LR, Brebi-Mieville P, Ili-Gangas C, Lebron C, Hernández-Arroyo M, Witter FR, Apelberg BJ, Roystacher M, Jaffe A, Halden RU, Sidransky D (2010) Global DNA hypomethylation is associated with *in utero* exposure to cotinine and perfluorinated alkyl compounds. Epigenetics 5:539–546
304. Alexander BH, Olsen GW, Burris JM, Mandel JH, Mandel JS (2003) Mortality of employees of a perfluorooctanesulphonyl fluoride manufacturing facility. Occup Environ Med 60: 722–729
305. Alexander BH, Olsen GW (2007) Bladder cancer in perfluorooctanesulfonyl fluoride manufacturing workers. Ann Epidemiol 17:471–478
306. Grice MM, Alexander BH, Hoffbeck R, Kampa DM (2007) Self-reported medical conditions in perfluorooctanesulfonyl fluoride manufacturing workers. J Occup Environ Med 49: 722–729
307. Olsen GW, Burlew MM, Marshall JC, Burris JM, Mandel JH (2004) Analysis of episodes of care in a perfluorooctanesulfonyl fluoride production facility. J Occup Environ Med 46:837–846
308. Olsen GW, Zobel LR (2007) Assessment of lipid, hepatic, and thyroid parameters with serum perfluorooctanoate (PFOA) concentrations in fluorochemical production workers. Int Arch Occup Environ Health 81:231–246
309. Lundin JI, Alexander BH, Olsen GW, Church TR (2009) Ammonium perfluorooctanoate production and occupational mortality. Epidemiology 20:921–928
310. Costa G, Sartori S, Consonni D (2009) Thirty years of medical surveillance in perfluorooctanoic acid production workers. J Occup Environ Med 51:364–372
311. Butenhoff JL, Gaylor DW, Moore JA, Olsen GW, Rodricks J, Mandel JH, Zobel LR (2004) Characterization of risk for general population exposure to perfluorooctanoate. Regul Toxicol Pharmacol 39:363–380
312. Mundt DJ, Mundt KA, Luippold RS, Schmidt MD, Farr CH (2007) Clinical epidemiological study of employees exposed to surfactant blend containing perfluorononanoic acid. Occup Environ Med 64:589–594
313. Fei C, McLaughlin JK, Lipworth L, Olsen J (2009) Maternal levels of perfluorinated chemicals and subfecundity. Hum Reprod 24:1200–1205
314. Fei C, McLaughlin JK, Tarone RE, Olsen J (2008) Fetal growth indicators and perfluorinated chemicals: a study in the Danish National Birth Cohort. Am J Epidemiol 168:66–72
315. Washino N, Saijo Y, Sasaki S, Kato S, Ban S, Konishi K, Ito R, Nakata A, Iwasaki Y, Saito K, Nakazawa H, Kishi R (2009) Correlations between prenatal exposure to perfluorinated chemicals and reduced fetal growth. Environ Health Perspect 117:660–667
316. Monroy R, Morrison K, Teo K, Atkinson S, Kubwabo C, Stewart B, Foster WG (2008) Serum levels of perfluoroalkyl compounds in human maternal and umbilical cord blood samples. Environ Res 108:56–62
317. Nolan LA, Nolan JM, Shofer FS, Rodway NV, Emmett EA (2009) The relationship between birth weight, gestational age and perfluorooctanoic acid (PFOA)-contaminated public drinking water. Reprod Toxicol 27:231–238
318. Hamm MP, Cherry NM, Chan E, Martin JW, Burstyn I (2010) Maternal exposure to perfluorinated acids and fetal growth. J Exp Sci Environ Epidemiol 20:589–597
319. Stein CR, Savitz DA, Dougan M (2009) Serum levels of perfluorooctanoic acid and perfluorooctane sulfonate and pregnancy outcome. Am J Epidemiol 170:837–846
320. Nolan LA, Nolan JM, Shofer FS, Rodway NV, Emmett EA (2010) Congenital anomalies, labor/delivery complications, maternal risk factors and their relationship with perfluorooctanoic acid (PFOA)-contaminated public drinking water. Reprod Toxicol 29:147–155
321. Fei C, McLaughlin JK, Lipworth L, Olsen J (2010) Prenatal exposure to PFOA and PFOS and risk of hospitalization for infectious diseases in early childhood. Environ Res 110: 773–777

322. Fei C, McLaughlin JK, Lipworth L, Olsen J (2008) Prenatal exposure to perfluorooctanoate (PFOA) and perfluorooctanesulfonate (PFOS) and maternally reported developmental milestones in infancy. Environ Health Perspect 116:1391–1395
323. Fei C, Olsen J (2011) Prenatal exposure to perfluorinated chemicals and behavioral or coordination problems at age 7 years. Environ Health Perspect 119:573–578
324. Christensen KY, Maisonet M, Rubin C, Holmes A, Calafat AM, Kato K, Flanders WD, Heron J, McGeehin MA, Marcus M (2011) Exposure to polyfluoroalkyl chemicals during pregnancy is not associated with offspring age at menarche in a contemporary British cohort. Environ Int 37:129–135
325. Steenland K, Tinker S, Frisbee S, Ducatman A, Vaccarino V (2009) Association of perfluorooctanoic acid and perfluorooctane sulfonate with serum lipids among adults living near a chemical plant. Am J Epidemiol 170:1268–1278
326. Frisbee SJ, Shankar A, Knox SS, Steenland K, Savitz DA, Fletcher T, Ducatman AM (2010) Perfluorooctanoic acid, perfluorooctanesulfonate, and serum lipids in children and adolescents: results from the C8 Health Project. Arch Pediatr Adolesc Med 164:860–869
327. MacNeil J, Steenland NK, Shankar A, Ducatman A (2009) A cross-sectional analysis of type II diabetes in a community with exposure to perfluorooctanoic acid (PFOA). Environ Res 109:997–1003
328. Steenland K, Tinker S, Shankar A, Ducatman A (2010) Association of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) with uric acid among adults with elevated community exposure to PFOA. Environ Health Perspect 118:229–233
329. Lin CY, Chen PC, Lin YC, Lin LY (2009) Association among serum perfluoroalkyl chemicals, glucose homeostasis, and metabolic syndrome in adolescents and adults. Diabetes Care 32:702–707
330. Lin CY, Lin LY, Chiang CK, Wang WJ, Su YN, Hung KY, Chen PC (2009) Investigation of the associations between low-dose serum perfluorinated chemicals and liver enzymes in US adults. Am J Gastroenterol 105:1354–1363
331. Nelson JW, Hatch EE, Webster TF (2010) Exposure to polyfluoroalkyl chemicals and cholesterol, body weight, and insulin resistance in the general U.S. population. Environ Health Perspect 118:197–202
332. Melzer D, Rice N, Depledge MH, Henley WE, Galloway TS (2010) Association between serum perfluorooctanoic acid (PFOA) and thyroid disease in the U.S. National Health and Nutrition Examination Survey. Environ Health Perspect 118:686–692
333. Hoffman K, Webster TF, Weisskopf MG, Weinberg J, Vieira VM (2010) Exposure to polyfluoroalkyl chemicals and attention deficit hyperactivity disorder in U.S. children aged 12-15 years. Environ Health Perspect 118:1762–1767
334. Joensen UN, Bossi R, Leffers H, Jensen AA, Skakkebaek NE, Jørgensen N (2009) Do perfluoroalkyl compounds impair human semen quality? Environ Health Perspect 117: 923–927
335. Eriksen KT, Sørensen M, McLaughlin JK, Lipworth L, Tjønneland A, Overvad K, Raaschou-Nielsen O (2009) Perfluorooctanoate and perfluorooctanesulfonate plasma levels and risk of cancer in the general Danish population. J Natl Cancer Inst 101:605–609
336. Zhao B, Hu GX, Chu Y, Jin X, Gong S, Akingbemi BT, Zhang Z, Zirkin BR, Ge RS (2010) Inhibition of human and rat 3β -hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase 3 activities by perfluoroalkylated substances. Chem Biol Interact 188: 38–43

Toxicologically Relevant Phthalates in Food

Oliver Kappenstein, Bärbel Vieth, Andreas Luch, and Karla Pfaff

Abstract Various phthalates have been detected in a wide range of food products such as milk, dietary products, fat-enriched food, meat, fish, sea food, beverages, grains, and vegetables as well as in breast milk. Here we present an overview on toxicologically considerable phthalate levels in food reported in the literature. The most common phthalates detected are di-(2-ethylhexyl) phthalate (DEHP), di-*n*-butyl phthalate (DnBP), and di-isobutyl phthalate (DiBP). Milk analyses demonstrate that background levels in unprocessed milk are usually low. However, during processing the phthalate contents may significantly increase due to migration from plastic materials in contact with food. Among dietary products fat-enriched food such as cheese and cream were identified with highest levels of DEHP. Plasticized PVC from tubes, conveyor belts, or disposable gloves used in food processing is an important source for contamination of food, especially of fatty food. Paper and cardboard packaging made from recycled fibers are another important source of contamination. In addition, gaskets used in metal lids for glass jars have been identified as possible source for the contamination of foodstuffs with phthalates. The highest concentrations of DEHP reported (>900 mg kg^{-1}) were detected in food of high fat content stored in such glass jars. Beyond classical food, DEHP and DnBP were identified in human breast milk samples as the main phthalate contaminants. Phthalate monoesters and some oxidative metabolites were also quantified in breast milk.

Keywords Contaminants · Di-isobutyl phthalate · Di-*n*-butyl phthalate · Di-(2-ethylhexyl) phthalate · Food · Human breast milk · Packaging · Phthalates · Tolerable daily intake

O. Kappenstein (✉), B. Vieth, A. Luch, and K. Pfaff
Department of Product Safety, German Federal Institute for Risk Assessment (BfR),
Max-Dohrn-Strasse 8-10, 10589 Berlin, Germany
e-mail: Oliver.Kappenstein@bfr.bund.de

A. Luch (ed.), *Molecular, Clinical and Environmental Toxicology*,
Experientia Supplementum 101, DOI 10.1007/978-3-7643-8340-4_4,

List of Abbreviation

BBP	Benzyl-butyl phthalate
DEHP	Di-(2-ethylhexyl) phthalate
DEP	Di-ethyl phthalate
DiBP	Di-isobutyl phthalate
DiDP	Di-isodecyl phthalate
DiNP	Di-isononyl phthalate
DnBP	Di-*n*-butyl phthalate
DMP	Di-methyl phthalate
DnNP	Di-*n*-nonyl phthalate
DnOP	Di-*n*-octyl phthalate
LOD	Limit of detection
LOQ	Limit of quantification
MnBP	Mono-*n*-butyl phthalate
MBzP	Mono-benzyl phthalate
MCPP	Mono-3-carboxypropyl phthalate
MEHHP	Mono-2-ethyl-5-hydroxyhexyl phthalate
MEHP	Mono-ethylhexyl phthalate
MEOHP	Mono-2-ethyl-5-oxohexyl phthalate
MEP	Mono-ethyl phthalate
MiBP	Mono-isobutyl phthalate
MnBP	Mono-*n*-butyl phthalate
MiNP	Mono-isononyl phthalate
MMP	Mono-methyl phthalate
MnOP	Mono-*n*-octyl phthalate
n	Number of samples
NA	Not available
ND	Not detectable
PVC	Polyvinyl chloride
TDI	Tolerable daily Intake

Introduction

Phthalates, i.e., 1,2-benzene dicarboxylic acid esters, are used in a broad variety of industrial applications. This widespread use results in a ubiquitous distribution of phthalates in the general environment as well as in unwrought food materials [1–3]. Additional contamination may then occur during production, processing, and packaging of food [4]. Typical food contact materials that may release phthalates into foodstuffs are plasticized PVC, nitrocellulose, or synthetic rubbers.

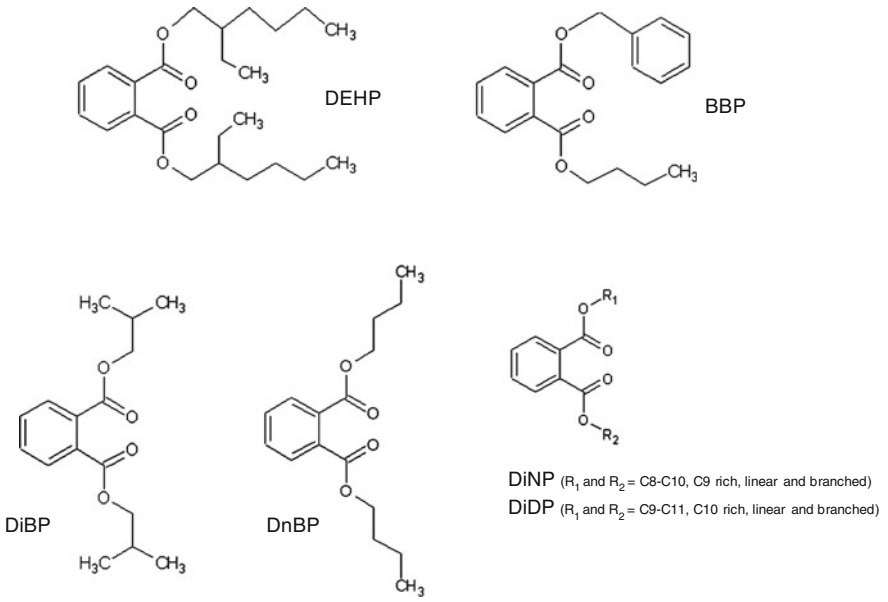


Fig. 1 Chemical structures of toxicologically relevant phthalates in food. *DEHP* di-(2-ethylhexyl) phthalate, *BBP* benzyl-butyl phthalate, *DnBP* di-*n*-butyl phthalate, *DiBP* di-isobutyl phthalate, *DiNP* di-isononyl phthalate, *DiDP* di-isodecyl phthalate

Table 1 List of common phthalates in food and corresponding tolerable daily intake (TDI) values

Phthalate	Abbreviation	CAS No.	TDI	References
Di-(2-ethylhexyl) phthalate	DEHP	117-81-7	0.05 mg kg^{-1} bw	[5]
Di-*n*-butyl phthalate	DnBP	84-74-2	0.01 mg kg^{-1} bw	[6]
Di-isobutyl phthalate	DiBP	84-69-5	–	
Di-isodecyl phthalate	DiDP	26761-40-0	0.15 mg kg^{-1} bw	[7]
Di-isononyl phthalate	DiNP	68515-48-0	0.15 mg kg^{-1} bw	[8]
Benzyl-butyl phthalate	BBP	85-68-7	0.5 mg kg^{-1} bw	[9]

TDI tolerable daily intake (milligram per kilogram body weight: mg kg^{-1} bw)

High amounts of phthalates (up to 40% or even more) are added to these materials in order to achieve the plasticizing effect.

Food is one of the most important sources through which humans are exposed to phthalates. The main goal of this article is to critically review existing literature regarding the occurrence of phthalate-like plasticizers in food, including breast milk, and to summarize the current state of knowledge. In addition, possible reasons for phthalate contamination of food are discussed.

Among all phthalates known to date, six commercially important compounds are considered in this review (Fig. 1). Of this group, five are being evaluated for their application in food contact materials by the European Food Safety Authority (EFSA), and tolerable daily intake (TDI) values were derived (Table 1).

Human Milk

Human milk is the most important food for neonates and infants. Fetuses and neonates are regarded as particularly susceptible to reproductive and developmental effects of phthalates [10]. Data on phthalates in human milk provide important information both on the overall exposure and uptake of adults (i.e., pregnant women and/or breastfeeding mothers) and on the intake of these contaminants by breast-fed infants. Numerous studies demonstrated the contamination of breast milk with phthalates [11–16]. The pattern of phthalate contamination of human milk is similar for all countries investigated, with DEHP being the predominant compound followed by DnBP. A compilation of data extracted from recent literature is provided in Table 2.

In Germany, first data on phthalates in human milk were reported in 1998 [12]. DEHP was found in all samples with concentrations between 71 and 160 μg kg^{-1}. DnBP emerged at lower concentrations (33–51 μg kg^{-1}). By contrast, other phthalates such as diethyl phthalate (DEP), benzyl-butyl phthalate (BBP), di-*n*-octyl phthalate (DnOP), and short-chain phthalates were not detected in human milk. These findings were confirmed in 2000 by additional German data [13]. Among 15 different phthalates investigated (including DEHP, DnBP, DEP, DiBP, DnOP, DnNP, and BBP) only DEHP and DnBP were detected. The reported levels were from 10 to 110 μg kg^{-1} and 50 μg kg^{-1}, respectively. A human milk sample from Norway, analyzed in 2001, supported the above-mentioned pattern and levels [15]. In a German follow-up study, DEHP was quantified in only four out of ten breast milk samples at 10 μg kg^{-1}, whereas DiBP and DnBP were not detected [11]. Even though the number of samples included in these German studies is very low, these data might indicate a trend over time toward lower levels of DEHP and DnBP in human milk, at least in Germany, which also would be expected in view of a slightly decreasing total phthalate exposure registered over the last years [10].

Applying more sensitive analytics, in 2005 the Swedish Environmental Protection Agency published data on DEHP and DnBP, but also on DEP, BBP, and DnOP [14]. Here, along with DEHP, BBP (mean value: 3 μg kg^{-1}) was identified in all samples, thus contrasting the results of all other studies that did not detect BBP. The mean value of DEHP in human milk was found comparable to German levels (30 μg kg^{-1}), reaching a maximum value of 305 μg kg^{-1}. DnBP, DEP, and DnOP were detected in only 20–30% of the samples with mean levels between 1.6 and 3.8 μg kg^{-1}. In addition, blood samples collected from breast milk donating mothers were included in the study. The results indicate that the less lipophilic short-chain phthalates, e.g., DEP and DnBP, mainly retained in the blood compartment.

Phthalate patterns in human milk from Canada were found to shift to distinctly higher DEHP and lower DnBP concentrations when compared to European samples. Zhu *et al.* detected DEHP and DnBP in all samples collected between 2003 and 2004 ($n = 86$) [16]. The mean value of 222 μg kg^{-1} DEHP is about tenfold higher than the DEHP concentration in European human milk collected during a similar time period [16]. In contrast, the mean concentration of DnBP was

Table 2 Levels of phthalate diesters detected in human milk

Country, year	DEP (μg kg^{-1})		BBP (μg kg^{-1})		DiBP (μg kg^{-1})		DnBP (μg kg^{-1})		DEHP (μg kg^{-1})		DnOP (μg kg^{-1})		References
	Mean (range)	n > LOQ	Mean (range)	n > LOQ	Mean (range)	n > LOQ	Mean (range)	n > LOQ	Mean (range)	n > LOQ	Mean (range)	n > LOQ	
Germany, 1998	ND	0/5	ND	0/5			(<20–51)[a]	3/5[a]	93 (71–160)	5/5	ND	0/5	[12]
Germany, 2000	ND	0/5	ND	0/5	ND	0/5	28 (10–50)	5/5	34 (10–110)	5/5	ND		[13]
Germany, 2004					ND (<10)	0/10	ND (<10)	0/10	<10 (<10–10)	4/10			[11]
Norway, 2001	ND	0/1	ND	0/1			8	1/1	10	1/1			[15]
Sweden, 2005	1.6 (0.22–2.6)	8/42	3.0 (0.06–4.5)	42/42			3.8 (1.5–20)	12/42	30 (0.42–305)	42/42	3.0 (0.24–13)	10/42	[14]
Canada, 2003–2004	0.31 (<0.21–8.1)	15/86	ND	0/86			0.87 (0.12–11)	85/86	222 (≤2920)	86/86	ND	0/86	[16]

[a]No differentiation between DiBP and DnBP

determined at 0.9 μg kg^{-1} and hence at distinctly lower levels as found for European samples. In addition, traces of DEP were detected in a small number of samples, but di-methyl phthalate (DMP), BBP, and DnOP were not. As yet there is no explanation for these large differences in DEHP and DnBP levels between Canada and Europe. The data might be interpreted as a result of different exposure patterns via products and foodstuffs in both parts of the world. Yet, DEHP and DnBP were the predominant phthalates in human breast milk in all studies published so far.

It has been well known that certain factors like the age of the mother or the length and number of nursing periods have an impact on the body burden of persistent organochlorine compounds and, hence, on their levels in human milk. This rule does not apply to phthalate esters due to their short elimination half-lives. Correspondingly, the changes in phthalate concentrations during a breast feeding period follow a different time pattern than those of persistent organochlorine compounds. Considerable fluctuations of DEHP and significant increases of DnBP concentrations were observed during a 6 months breast feeding period. These variations over time may be caused by short time day-to-day exposures of the individual mothers [16].

Phthalate diesters are rapidly metabolized, primarily into their hydrophilic monoesters. In case of high-molecular-weight phthalates, like DEHP, further enzymatic oxidation at the remaining alkyl chain results in even more hydrophilic metabolites. So, in addition to phthalate diesters, monoesters and some oxidative metabolites were identified in human milk [14, 17–19]. Usually, monoesters emerge as nonconjugated species in human milk, but as conjugates in serum, a fact that may be attributed to the more lipophilic character of the free metabolites compared to the corresponding glucuronides [18, 19]. In accordance with corresponding diester levels in breast milk, the mean levels of mono-ethylhexyl phthalate (MEHP) were generally higher than those of mono-*n*-butyl phthalate (MnBP). Surprisingly, mono-isononyl phthalate (MiNP) was the predominant monoester in some studies [17–19], although data on di-isononyl phthalate (DiNP) have not been reported in human milk up to now. The data on monoester metabolites are summarized in Table 3.

In some 130 breast milk samples from Finland and Denmark collected 1–3 months after birth, six monoesters were detected in almost all samples. Median concentrations of MiNP, MEHP, and MnBP in samples from Finland and Denmark were found at 89 and 101 μg kg^{-1}, 1.3 and 9.5 μg kg^{-1}, and 1.2 and 4.3 μg kg^{-1}, respectively. The levels of the less lipophilic monoesters mono-benzyl phthalate (MBzP), mono-ethyl phthalate (MEP), and mono-methyl phthalate (MMP) were considerably lower [17, 18]. In three pooled human milk samples from the USA, lower levels of MiNP were found, and levels similar to those in Europe were found for MEHP and MnBP (7.8 and 1.3 μg kg^{-1}, respectively) [19]. Furthermore, the oxidized metabolites mono-3-carboxypropyl phthalate (MCPP), mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), and mono-2-ethyl-5-oxohexyl phthalate (MEOHP) were identified in all three pooled samples with concentrations below the limit of quantification (LOQ: 0.2–0.5 μg kg^{-1}). Other monoesters such as

Table 3 Levels of phthalate monoesters detected in human milk

Country, year	Number of samples, *n*	MMP (μg kg^{-1}) Mean (range)	MEP (μg kg^{-1}) Mean (range)	MnBP (μg kg^{-1}) Mean (range)	MiBP (μg kg^{-1}) Mean (range)	MBzP (μg kg^{-1}) Mean (range)	MEHP (μg kg^{-1}) Mean (range)	MiNP (μg kg^{-1}) Mean (range)	References
Finland, 1997–2001	65	0.09[a] (<0.01–0.37)	0.97[a] (0.25–41.4)	12[a] (2.4–123)		1.3[a] (0.4–26)	13[a] (4.0–1410)	89[a] (28–230)	[17]
Denmark, 1997–2001	65	0.1[a] (<0.01–5.53)	0.93[a] (0.07–33.6)	4.3[a] (0.6–10900)		0.9[a] (0.2–14)	9.5[a] (1.5–191)	101[a] (27–469)	[17, 18]
Sweden, 2005	42	ND (<1.0)	ND (<1.0)	2.4 (<1.1–6)	1.8 (<1.0–6.8)	1.8 (<1.0–6.8)	2.6 (<1.0–7.2)	ND (<1.0)	[14]
USA, 2004	3 Pools	ND (<0.2)	ND (1.9)	1.3	ND (<0.3)	ND (<0.5)	7.8	16.1	[19]

[a]Median value

MMP, MEP, MBzP, mono-isobutyl phthalate (MiBP), and mono-*n*-octyl phthalate (MnOP) were not verified.

The studies described above demonstrate that metabolites of the long-chain phthalates MiNP and MEHP are predominant in human milk, whereas the short-chain compounds MEP and MMP are only minor. Presumably this result mirrors an increasing fat solubility of longer aliphatic chain phthalates.

In the Swedish study phthalate diesters, monoesters and corresponding oxidative metabolites were monitored in the very same breast milk samples [14]. In contrast to the data reported from Finland, Denmark, and the USA, MiNP was not detectable. MEHP and MnBP were quantified in 25–35% of the samples with similar mean concentrations (2.6 and 2.4 μg kg^{-1}, respectively). MiBP and MBzP were detected in a small number of samples, whereas the concentrations of the monoesters MMP, MEP, and MnOP as well as those of the oxidative metabolites MCPP, MEHHP, and MEOHP were below the detection limit (1 μg kg^{-1}) without exception. In general, levels of metabolites were lower compared to those of parent compounds. In addition, levels of diesters and their corresponding monoesters did not correlate, what could be due to the low number of samples found above the limit of detection (LOD).

Breast feeding is the most important kind of nutrition for newborns and infants. Based on the mean and maximum concentrations reported for DnBP and DEHP, the intake by breast feeding for a 1-month-old infant (mean body weight: 4.15 kg, mean breast milk consumption: 645 ml) has been calculated and summarized in Table 4. By comparing calculated intakes with TDI values for DnBP (10 μg kg^{-1} bw) and DEHP (50 μg kg^{-1} bw) derived by EFSA (see Table 1), it becomes clear that extremely high DEHP levels in Canadian human milk may result in an intake far beyond the accepted tolerable intake. In Europe, however, breast feeding is not expected to contribute to a higher-than-tolerated daily intake and therefore should be widely supported and encouraged.

Milk and Milk Products

In contrast to human milk, bovine milk is more likely to be contaminated via environmental sources and processing operations. Here, phthalates may be migrating from plasticized tubes into milk and subsequently into all kinds of milk products.

Table 4 Body intake of DnBP and DEHP via breast feeding for a 1-month-old infant

Country, year	DnBP (μg kg^{-1} bw per day)		DEHP (μg kg^{-1} bw per day)	
	Mean	Maximum	Mean	Maximum
Germany, 1998 [12]		7.9	14.5	24.9
Germany, 2000 [13]	4.4	7.8	5.3	17.1
Germany, 2004 [11]	<1.6	<1.6	<1.6	1.6
Norway, 2001 [15]	1.2		1.6	
Sweden, 2005 [14]	0.6	3.1	4.7	47.4
Canada, 2003–2004 [16]	0.1	1.7	34.5	453.8

bw body weight

To assess the extent of DEHP migration from plasticized tubing used in commercial milking equipment, milk samples were collected at different stages of the milking process in the UK in 1990 [20]. Milk samples obtained by hand milking contained only low DEHP levels (0.005 mg kg^{-1}). By contrast, machine milking resulted in a tenfold increase in DEHP concentrations; values in individual milking chambers averaged at 0.03 mg kg^{-1} and increased to 0.05 mg kg^{-1} in the central collecting tank.

In 1991, DEHP contents in retail whole milk samples from German ($n = 1$) and Danish ($n = 14$) dairies were similar. In this kind of source, mean concentrations of DEHP were found below 0.05 mg L^{-1} [21].

Since phthalates are lipophilic migration rates may depend on the fat content of the food. This assumption is supported by data on DEHP in milk, cream, butter, and cheese, collected in various European countries [22]. During processing, the DEHP level in cream piles up to 1.93 mg kg^{-1}, whereas low fat milk contained only <0.01–0.07 mg kg^{-1}. Retail dairy products were found contaminated with <0.01–0.55 mg kg^{-1} DEHP, but composite milk samples from doorstep delivery were only low (<0.01–0.09 mg DEHP kg^{-1}). Retail samples of cheese, butter, and other fatty milk products varied considerably in DEHP levels. The highest concentration of 16.8 mg kg^{-1} was found in a sample of cheese. In this study ($n = 25$), average concentrations in cheese were between 0.6 and 3.0 mg kg^{-1} DEHP and thus similar to cream samples with 0.2–2.7 mg kg^{-1} DEHP ($n = 20$) [22].

The influence of packaging on the phthalate levels in milk became obvious from investigations made in Spain in 2004 [23]. Processed ultra-heat-treated (UHT) whole milk packaged in Tetra Brik and sterilized milk in high-density polyethylene (HDPE) bottles were analyzed. Among other phthalates, all samples contained DnBP in concentrations of up to 0.05 mg kg^{-1} and DEHP in concentrations between 0.015 and 0.025 mg kg^{-1}.

In 2006, a survey in Denmark reported that DnBP, BBP, DiNP, and di-isodecyl phthalate (DiDP) were not detectable in milk and dairy products including infant formula [24]. The concentrations of DEHP measured (e.g., raw milk: 0.007–0.030 mg kg^{-1}; $n = 18$) indicate a low background level of DEHP in milk. This study also demonstrated that phthalate concentrations in raw milk and in processed products with a comparable fat content were about the same.

Collected data from Canada, USA, and Japan for the year 2003 revealed mean concentrations of 0.04 mg kg^{-1} DBP ($n = 12$) and 1.5 mg kg^{-1} DEHP ($n = 107$), respectively, in dairy products (excluding milk) [25]. In milk, mean concentrations of 0.012 mg kg^{-1} DnBP ($n = 50$) and 0.08 mg kg^{-1} DEHP ($n = 108$) were measured.

Over a period of 15 years (1990–2006), several publications from Europe, America, and Japan regularly reported data mainly on DEHP concentrations and occasionally also on DnBP measured in milk and dairy products. However, significant decreases of DnBP and DEHP concentrations in milk were not observed. According to these data, phthalate concentrations found in milk and milk products in principle depend on fat contents and manufacturing processes (Table 5).

Table 5 Concentrations of DEHP in milk and milk products

Food	*n*	DEHP (mg kg^{-1})		References	Country, year
		min	max		
Milk and milk products with a fat content of up to 5%	3	0.010	0.100	[26]	Canada, 1995
	12	0.008	0.242	[27]	Canada, 2005
	14	<0.005	0.095	[20]	Norway, 1990
	15	<0.1	<0.14	[22]	Denmark, 1991
	9	0.010	0.150	[28]	Austria, 1994
	10	0.300		[29]	UK, 1996
	11	0.010	0.110	[11]	Germany, 1999
	4	0.015	0.027	[23]	Spain, 2004
	25	0.007	0.037	[24]	Denmark, 2006
Cream and milk products with a fat content between 7 and 35%	3	0.070	1.200	[26]	Canada, 1995
	1	0.120	0.140	[20]	Norway, 1990
	17	0.200	2.700	[21]	Norway, 1994
	6	0.180	0.320	[11]	Germany, 1999
Cheese	4	nd		[29]	UK, 1996
Hard/sliced cheese	16	0.300	5.500	[26]	Canada, 1995
	25	0.200	16.800	[21]	Norway, 1994
	10	0.120	0.920	[11]	Germany, 2004
Soft and cream cheese	4	0.070	2.100	[26]	Canada, 1995

Baby Food and Infant Formulae

In 1996, studies on DiBP, DnBP, and DEHP contents in composite retail infant formula samples ($n = 12$) were conducted in the UK [29]. Among all phthalates investigated, DEHP was most abundant and has been identified in all samples. The concentrations of DEHP (0.33–0.98 mg kg^{-1}), DiBP (0.06–0.26 mg kg^{-1}), and DnBP (0.08–0.40 mg kg^{-1}) were always found in the same range, irrespective of the kind of formula investigated, i.e., casein-dominant, wheat-dominant, or soya-based.

During a follow-up survey in 1998 phthalate concentrations were considerably lower [30]. Only DnBP, DEHP, and BBP were detected and quantified in the range of 0.01–0.44 mg kg^{-1} in the 39 samples of infant formulae (powdered and ready to feed) investigated.

In 2000, DnBP, DEHP, and BBP were analyzed in 11 samples of baby food and reconstituted infant formulae, derived from the Danish market [31]. In one sample of baby food, DnBP and BBP were determined simultaneously (0.04 and 0.005 mg kg^{-1}, respectively). In two samples of infant formula, BBP was quantified at 0.004 and 0.01 mg kg^{-1}. DEHP was determined in two samples of baby food (0.36 and 0.63 mg kg^{-1}) and two samples of infant formulae (0.04 and 0.06 mg kg^{-1}). In 2003, the compilation of data from Canada, USA, and Japan revealed mean concentrations of 0.07 mg kg^{-1} DnBP ($n = 53$) and 0.2 mg kg^{-1} DEHP ($n = 66$) in powdered infant formulae, 0.003 mg kg^{-1} DnBP ($n = 4$) and 0.007 mg kg^{-1} DEHP ($n = 9$) in liquid infant formulae, and 0.03 mg kg^{-1} DnBP ($n = 12$) and 0.12 mg kg^{-1} DEHP ($n = 16$) in baby food, respectively [25]. In 2006, a survey in Denmark reported amounts of DEHP in the range of 0.037–0.138 mg kg^{-1}

($n = 6$) in reconstituted infant formula delivered from different parts of the world and 0.010–0.023 mg kg^{-1} ($n = 2$) in liquid infant formula [24].

The presented data on baby food and different kinds of infant formulae consistently demonstrate that DnBP and DEHP are the prevailing phthalates in baby food and infant formulae. In addition, the values reported in the literature indicate that DEHP concentrations were slightly decreasing over the past decade.

Food with High Fat Content

Due to the high lipid solubility of phthalates fat-rich food is of special importance in terms of contamination. DEHP and DnBP concentrations measured in adipose tissues of farm animals are summarized in Table 6. There was a statistically significant correlation between DEHP and DnBP levels in the animal's fat tissue and in their feed [32].

In 2002, a survey in Japan determined DnBP, DEHP, and BBP in processed foods like ham and sausages ($n = 8$), fried dumplings, shao-mai ($n = 8$), and fried fishes ($n = 6$) [33]. The levels of DnBP, DEHP, and BBP found were up to 0.048, 0.749, and 0.006 mg kg^{-1}, respectively. As part of a German retail market survey in 2004, samples of minced meat and ham sausage were analyzed for their contents of DnBP, DiBP, and DEHP [11]. One out of five samples contained 0.28 mg kg^{-1} DEHP. Another sample contained 0.13 mg kg^{-1} DnBP. Wrapped ham sausages from different producers showed DEHP levels between 0.11 and 0.16 mg kg^{-1} in three out of five samples. By contrast, DnBP and DiBP were not detectable.

Phthalate contaminations occur not only in animal fat tissue, but also in vegetable oil. In 2005, the German foundation for comparative product testing ("Stiftung Warentest") reported results of a survey on DEHP, BBP, DiNP, and DiDP contents in 25 "native extra" olive oils [34]. The highest level of DEHP contamination reported was 75 mg kg^{-1}. Another sample was contaminated with 9.3 mg kg^{-1} DEHP and 40 mg kg^{-1} DiDP.

Fish and Fish Products

There are only a few data published on phthalates in aquatic food that indicate a broad variability in phthalate contamination. In 2002, studies in Japan [33] determined DnBP, DEHP, and BBP levels in nine fish paste products with concentrations

Table 6 Concentrations of DEHP and DnBP in adipose tissue of animals (according to [32])

Sample	DEHP (mg kg^{-1})	DnBP (mg kg^{-1})
Bovine fat	0.55–1.52	1.76–4.17
Porcine fat	0.20–0.80	1.37–6.12
Poultry fat	0.20–1.71	0.20–0.68

of up to 0.043, 0.303, and 0.001 mg kg^{-1}, respectively. The route of phthalate contamination in fish paste products has not been clarified.

Low levels of DnBP and DEHP were found in a Dutch study on aquatic wildlife in 2006 [35]. In this study, 25 samples of bream and roach led to 15 positive results for DEHP with a mean concentration of 0.002 mg kg^{-1}. In 18 out of 25 samples, DnBP was quantified with a mean value of 0.002 mg kg^{-1}. Higher levels were found in freshwater carps from Austrian ($n = 3$), Czech ($n = 3$), Polish ($n = 3$), and Slovenian ($n = 3$) waters in 2005 [36]. Concentrations ranged from 0.3 to 1.1 mg kg^{-1} DnBP, 1.0 to 8.8 mg kg^{-1} DiBP, 0.1 to 0.8 mg kg^{-1} DEHP, and <0.1 to 8.9 mg kg^{-1} DiNP, respectively. Collected data from different countries showed no significant differences for those phthalates listed.

The reported data indicate a tendency to higher phthalate levels in farmed fish when compared to wild fish. The impact of fish feed on phthalate concentrations in farmed fishes has not yet been clarified.

Grain, Vegetables, and Nuts

Due to the lack of data on phthalate contents, proper exposure assessment for staple foods (e.g., grain, vegetables, and related products) is challenging. Since staple foods are consumed in high amounts the need for risk assessment is obvious.

Six samples of sliced rye bread packed in plastic foil (e.g., polypropylene) were investigated. The DEHP levels ranged from 0.04 to 0.75 mg kg^{-1}. Conversely, for both DnBP and DiBP levels of 0.02 mg kg^{-1} were not exceeded [11]. Due to the large surface area per mass and a high fat content that both might result in higher absorption of phthalates, ground, and rasped hazelnuts were also included into analysis. The levels of DEHP ranged between 0.06 and 1.58 mg kg^{-1} ($n = 5$). DiBP and DnBP were detected only once with a concentration of 0.1 and 0.17 mg kg^{-1}, respectively [11]. In 2002, phthalates were determined in bread ($n = 5$), noodle ($n = 6$), and Korean pickles ($n = 5$) in a Japanese survey. DnBP, DEHP, and BBP were observed at levels of up to 0.020, 0.304, and 0.017 mg kg^{-1}, respectively [33].

Beverages

Due to the solubility properties of phthalates, phthalate contamination of nonalcoholic beverages usually is very low, whereas alcoholic beverages contain appreciable levels.

Studies conducted in 1995 found DEHP at concentrations of up to 0.492 mg L^{-1} in 47 vodka and flavored vodka samples and DBP at up to 0.204 mg L^{-1} in 18 out of 50 samples [37]. In 1998, these findings were confirmed by measuring DnBP and DEHP contents in retail beverages from the Japanese and Korean markets.

The highest concentrations in 24 tested beverages were 0.275 mg L^{-1} DnBP in red wine and 0.127 mg kg^{-1} DEHP in beer, whereas phthalate levels in examined bottled water ($n = 3$) and vinegar ($n = 2$) were below the LOD [38]. To substantiate these results, in 2007 Wolheim *et al.* examined various alcoholic beverages, i.e., wine, whiskey, and brandy, filled in different containers (e.g., glass, plastic tubes), for their contents on DnBP, DEHP, DiNP, and DiDP [39]. DnBP was detected only in 3 out of 33 samples with a maximum concentration of 1.9 mg L^{-1}, and DEHP in 4 out of 33 samples with a maximum concentration of 2.0 mg L^{-1}. By contrast, DiNP and DiDP were not found. Variations in phthalate concentrations depending on the kind of packaging were not observed. Based on those few samples positive for phthalates, a correlation between phthalate contents and alcohol percentage could not been derived.

In 2008, an additional study on DEHP, DiBP, DnBP, and BBP in 62 samples of red and white wine yielded maximum concentrations of 0.276, 0.260, 0.244, and 0.269 mg L^{-1}, respectively [40]. Statistical analysis of the results revealed significantly higher incidences and concentrations of DnBP and BBP in commercial compared to artisanal wines; DiBP and DEHP levels were similar in both groups [40].

Previous studies reported higher concentrations of DEHP in wine and whisky [41]. Maximum DEHP contents were determined at 3.6 mg L^{-1} (wine) and 6.8 mg L^{-1} (whisky). The data published indicated a ubiquitous occurrence of phthalates in those alcoholic beverages investigated.

In summary, analysis of DEHP and DnBP in alcoholic beverages demonstrated detectable levels in all types investigated, thereby encompassing a wide concentration range.

Contamination of Food with Phthalates Via Food Processing

In general, plasticized PVC articles used in food processing such as tubes, conveyor belts, or disposable gloves have been identified as important source for phthalate contamination of food.

The release of phthalates was investigated in 12 plastic components conventionally used in industrial extraction of citrus essential oils [42]. Significantly larger quantities of contaminants were released from new plastic parts than from reused material. Citrus essential oil produced via industrial extraction and application of plastic items contains up to 4.45 mg kg^{-1} DnBP, 62 mg kg^{-1} DiBP, and 29.9 mg kg^{-1} DEHP [42–44]. In these studies, qualitative and quantitative differences of plasticizers in oils were attributed to different types of plastic components used in the extraction process.

In 2000, a Japanese study identified PVC tubes used during production of baby food as a source of DEHP contamination (5.99 mg kg^{-1}). The contamination was eliminated after PVC had been replaced by stainless steel tubes [45]. Contaminations with DnBP, DEHP, BBP, and DiNP were further determined in 16 retail packed lunches and 10 set lunches, purchased in restaurants. In all samples, DEHP

was the predominant phthalate with concentrations up to 11.8 mg kg^{-1} (packed lunch) and up to 0.30 mg kg^{-1} (set lunch), respectively [46]. To determine the sources of phthalate contamination samples of the packaged lunches were taken from the factory after each individual step of preparation and separately analyzed for phthalates. For instance, fresh chicken that contained 0.08 mg kg^{-1} DEHP prior to processing was measured with 13.1 mg kg^{-1} after frying and 16.9 mg kg^{-1} DEHP after packaging. Further studies confirmed that an intensive contact of disposable PVC gloves with food during processing resulted in statistically higher DEHP concentrations in the end product [45, 47]. A total of 63 duplicate diet samples obtained from three Japanese hospitals over a period of 1 week were analyzed for DEHP, BBP, DiNP, and other phthalates in 1999 and 2001. In all samples, DEHP was present as the most abundant phthalate and found in a concentration range of 0.01–4.4 mg kg^{-1}.

To corroborate a link between DEHP contents in food and disposable PVC gloves used in the preparation of foods, samples of boiled rice, croquette, and boiled dry radish were handled in the laboratory with PVC gloves containing 30% w/w DEHP. As a result of this experiment, DEHP levels were determined at 0.05 mg kg^{-1} in rice, 0.33 mg kg^{-1} in croquette, and 11.1 mg kg^{-1} in radish. When alcohol was sprayed onto the gloves for disinfection, the migration of DEHP further increased to 2.03 mg kg^{-1} in rice, 2.45 mg kg^{-1} in croquette, and 18.4 mg kg^{-1} in radish.

Sauvegrain and Guinard investigated the impact of repeated contact of PVC gloves with fatty food on its contamination with phthalates (DnBP, DEHP, DiNP, and DnOP) [Sauvegrain (2001) Laboratoire National d'Essais (LNE) Paris, France, personal communication]. The phthalate levels were determined in salmon slices after the first and repeated contacts for 5 s with PVC gloves (Fig. 2). The results indicate that disposable PVC gloves plasticized with phthalates represent an important source for phthalate contamination of such kinds of foodstuff. Here, the

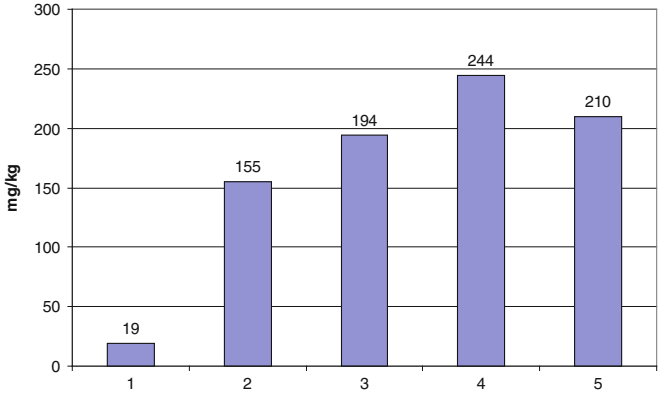


Fig. 2 Migration of phthalates from a disposable vinyl glove to fatty food. First contact (*1*), consecutive contacts after 4′55″ (*2*), 9′55′ (*3*), 14′55″ (*4*), and 19′55″ (*5*) [according to Sauvegrain (2001) Laboratoire National d'Essais (LNE) Paris, France, personal communication]

increase of phthalate levels in food is mainly caused by a thin fatty layer on the surface of the PVC glove left back after first contact with the fat-rich food. This layer of fatty material then serves as a lipophilic sink extracting phthalates from the glove and subsequently transferring them into the food during handling.

Phthalate Contamination Resulting from Paper and Cardboard Packaging

In addition to plasticized polymers such as PVC, phthalate contamination of food may also origin from paper and cardboard used as food packaging material. However, up to now only a few reports on phthalates in paper and cardboard packaging are available in the literature.

In 1999, a Finish study focused on paper packaging samples used for many kinds of foodstuffs and obtained directly from the manufacturer [48]. Half of the 29 paper and cardboard samples contained DEHP, DnBP, and DiBP in amounts exceeding 5 mg kg^{-1}. The maximum contents of DiBP and DnBP were 450 and 200 mg kg^{-1}, respectively. Phthalates were also determined in sugar before and after packaging. After 4 months of storage, migration of phthalates into the sugar ranged from 57 to 74% of the original content in the packaging material. Packed sugar contained 2.2–2.6 DiBP mg kg^{-1} and 0.5–1 mg kg^{-1} DnBP. There was a gradient of phthalate concentrations in sugar from the packaging material to the sugar in the center of the package [48].

Current data on the contents of DiBP and DnBP in food wrapped in folding boxes made out of recycled fibers were determined by Brauer (2008, Chemisches und Veterinäruntersuchungsamt [CVUA] Münsterland-Emscher-Lippe, Münster, Germany, personal communication). In 29 out of 61 samples, levels greater than 0.3 mg kg^{-1} DiBP could be detected (Table 7), with the maximum concentration of 5.0 mg kg^{-1} food. By contrast, DnBP did not exceed 0.23 mg kg^{-1} in food. These findings were linked to DiBP in dispersion glue that is applied in the manufacture of printed products, folding boxes, and corrugated board used for nonfood applications and other articles. Since it cannot be removed appropriately, DiBP reenters the paper cycle during recycling of these products. Fat-containing foods as well as powder or fine grain foods like rice, baking mixtures, or breadcrumbs are particularly at risk of containing high DiBP contamination [49].

Migration from Other Types of Food Packaging

In the last years, gaskets in metal lids for glass jars were found to be one of the most relevant sources for phthalate contamination of foodstuffs via packaging materials. However, and in contrast to the situation with paper and board packaging (see above), butyl phthalates are not used in the production of gaskets.

Table 7 DiBP and DnBP levels in food originating from paper and board packaging [according to Brauer (2008, Chemisches und Veterinäruntersuchungsamt (CVUA) Münsterland-Emscher-Lippe, Münster, Germany, personal communication)]

Kind of food	DiBP (mg kg^{-1} food)	DnBP (mg kg^{-1} food)
Couscous ($n = 1$)	1.10	0.11
Pearl barley ($n = 1$)	0.61	0.11
Rice ($n = 8$)	0.32–1.10	<0.02–0.22
Oat flakes ($n = 1$)	0.55–0.93	0.07–0.14
Cereal ($n = 1$)	0.40	0.09
Spelt ($n = 1$)	1.62	0.23
Flour and flour mixes ($n = 6$)	0.33–5.00	<0.02–0.07
Crisp bread ($n = 4$)	0.47–1.81	0.04–0.15
Icing sugar ($n = 1$)	3.16	0.04
Choko granules ($n = 1$)	0.38	<0.02
Table salt ($n = 1$)	0.48	0.08

In 2005, a survey on oily products in glass jars with metal closures (158 samples) was performed in the Canton of Zurich [50]. Within the gasket of the metal lids, DEHP was found in 43 samples (27% of the total), DiNP was also detected, and DiDP was found in 26 samples (16% of the total). Maximum concentrations in the food were 270 mg kg^{-1} for DiNP, 740 mg kg^{-1} for DiDP, and 825 mg kg^{-1} for DEHP, respectively. Asian seasoning pastes and sauces (25 samples) sold in glass jars with metal lids were examined for their contents of plasticizers in 2007 [51]. Eight samples (32%) contained DEHP amounts higher than 1.5 mg kg^{-1}, three of them beyond 300 mg kg^{-1}. DiNP was determined in nine samples (36%), of which seven samples contained more than 9 mg kg^{-1}, including four samples with more than 900 mg kg^{-1}. Two samples (8%) contained more than 9 mg kg^{-1} DiDP. In a survey from a German enforcement laboratory, phthalates were detected in 17 samples in a total of 51. Phthalate concentrations were up to 405 mg kg^{-1} for DiDP, 103 mg kg^{-1} for DiNP, and 195 mg kg^{-1} for DEHP, respectively [52].

Analytical Issues

A reliable survey of phthalate contamination in food is hampered by one major analytical issue. Fankhauser-Noti and Grob pointed out that the "blank problem" (background levels) in a laboratory is mainly caused by phthalate contamination of the air [53]. Therefore, absorption into solvents, glassware, and other labware and articles cannot be completely prevented.

Due to their ubiquitous occurrence in the laboratory, the major problem in phthalate analysis is the contamination of used chemicals and analytical equipment leading to false-positive results or overestimation. This "blank problem" results in a bias at each individual step of the entire analytical scheme including sampling, homogenization, sample extraction, sample preparation, and determination. Currently, there are only a few reports addressing the issue of background contamination by measuring blank values [24, 31, 40, 45, 54]. Any investigation intended to

estimate the level of phthalates in food should critically discuss the performance criteria of the analytical methods applied. Previous investigations of phthalates in food usually did not address the background problem sufficiently.

Conclusions

The number of European studies on phthalate levels in food is limited. Nevertheless, phthalates have been identified in all kinds of food. The reported concentrations of phthalates vary widely due to the dependence of values on the lipid content of the foodstuff investigated and the influence of different contamination pathways, e.g., processing, handling, and packaging, but also possibly because of analytical difficulties and insufficiencies. Due to their lipophilic nature, concentrations of phthalates in general are higher in fatty foods. Food can be contaminated through environmental sources and/or via processing and packaging. Considerably higher phthalate levels were found in food in contact to materials such as plasticized PVC used in various articles, e.g., tubes, conveyor belts, or disposable gloves. Food contact materials thus may represent an important contamination source, especially for food with higher fat contents. Overall, food is the dominating source of human exposure to DiBP, DnBP, and DEHP [55].

Recently, risk reduction measures were initiated by the European community. The Commission Regulation (EU) 10/2011 on materials and articles intended to come into contact with food stipulates restrictions on the use of BBP, DEHP, DnBP, DiNP, and DiDP as plasticizers in plastics intended to come into contact with foods. Phthalates are also present in paper and board produced from recycled fibers. Possible other sources are printing inks and adhesives from which substances may migrate into foods. However, measures regulating phthalates in these materials are currently absent.

More comprehensive monitoring of phthalates in food is urgently required. In particular, staple foods consumed in high amounts should be included into the range of foodstuffs to be analyzed. It is also important for proving the effectiveness of those regulatory measures initiated, as well as for identifying other contamination sources of food. Furthermore, there is still a lack of data on the potential carryover of phthalates from feed stuff to food of animal origin. Concurrently, future surveys need to consider the analytical blank problem appropriately through development and validation of harmonized protocols for methods that imply a lower risk of contamination.

References

1. European Union (2004) Risk assessment report: dibutyl phthalate. Addendum to the environmental section. CAS No: 84-74-2. EINECS No: 201-557-4. Summary risk assessment report. Online available at http://ecb.jrc.it/DOCUMENTS/Existing-Chemicals/RISK_ASSESSMENT/SUMMARY/dibutylphthalatesum003.pdf

2. European Union (2003) Risk assessment report: 1,2-benzenedicarboxylic acid, di-C8-10-branched alkyl esters, C9-rich and di-"Isononyl" phthalate (DiNP). CAS Nos: 68515-48-0 and 28553-12-0. EINECS Nos: 271-090-9 and 249-079-5. Online available at http://ecb.jrc.it/DOCUMENTS/Existing-Chemicals/RISK_ASSESSMENT/REPORT/dinpreport046.pdf
3. European Union (2003) Risk assessment report: 1,2-benzenedicarboxylic acid, di-C9-11-branched alkyl esters, C10-rich and di-"isodecyl" phthalate (DiDP). CAS Nos: 68515-49-1 and 26761-40-0. EINECS Nos: 271-091-4 and 247-977-1. Online available at http://ecb.jrc.it/DOCUMENTS/Existing-Chemicals/RISK_ASSESSMENT/REPORT/didpreport041.pdf
4. Grob K, Biedermann M, Scherbaum E, Roth M, Rieger K (2006) Food contamination with organic materials in perspective: packaging materials as the largest and least controlled source? A view focusing on the European situation. Crit Rev Food Sci Nutr 46:529–535
5. EFSA (2005) Opinion of the scientific panel on food additives, flavourings, processing aids and material in contact with food (AFC) on a request from the commission related to *bis*(2-ethylhexyl)phthalate (DEHP) for use in food contact materials. EFSA J 243:1–20 (adopted 23 June 2005)
6. EFSA (2005) Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food (AFC) on a request from the commission related to di-butylphthalate (DBP) for use in food contact materials. EFSA J 242:1–17
7. EFSA (2005) Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food (AFC) on a request from the commission related to di-isodecylphthalate (DIDP) for use in food contact materials. EFSA J 245:1–14 (adopted 30 July 2005)
8. EFSA (2005) Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food (AFC) on a request from the commission related to di-isononylphthalate (DINP) for use in food contact materials. EFSA J 244:1–18 (adopted 30 July 2005)
9. EFSA (2005) Opinion of the scientific panel on food additives, flavourings, processing aids and materials in contact with food (AFC) on a request from the commission related to butylbenzylphthalate (BBP) for use in food contact materials. EFSA J 241:1–14
10. Wittassek M, Koch HM, Angerer J, Brüning T (2011) Assessing exposure to phthalates—the human biomonitoring approach. Mol Nutr Food Res 55:7–31
11. Pfordt J (2004) Di(2-ethylhexyl)phthalat (DEHP) und Dibutylphthalate in einigen Lebensmitteln mit Kunststoffverpackungen und in Frauenmilch. Dtsch Lebensm Rundsch 11:431–436
12. Gruber L, Wolz G, Piringer O (1998) Untersuchungen von Phthalaten in Baby-Nahrung. Dtsch Lebensm Rundsch 94:177–179
13. Bruns-Weller E, Pfordt J (2000) Bestimmung von Phthalsäureestern in Lebensmitteln, Frauenmilch, Hausstaub und Textilien. UWSF—Z Umweltchem Ökotox 12:125–130
14. Högberg J, Hanberg A, Berglund M, Skerfving S, Remberger M, Calafat AM, Filipsson AF, Jansson B, Johansson N, Appelgren M, Håkansson H (2008) Phthalate diesters and their metabolites in human breast milk, blood or serum, and urine as biomarkers of exposure in vulnerable populations. Environ Health Perspect 116:334–339
15. Janak K, Ahmad I (2001) The new method for determination of phthalates in breast milk by HPLC-UV-MS with on-line sample processing. Organohalog Compds 50:45–48
16. Zhu J, Phillips SP, Feng YL, Yang X (2006) Phthalate esters in human milk: concentration variations over a 6-month postpartum time. Environ Sci Technol 40:5276–5281
17. Main KM, Mortensen GK, Kaleva MM, Boisen KA, Damgaard IN, Chellakooty M, Schmidt IM, Suomi AM, Virtanen HE, Petersen DV, Andersson AM, Toppari J, Skakkebaek NE (2006) Human breast milk contamination with phthalates and alterations of endogenous reproductive hormones in infants three months of age. Environ Health Perspect 114:270–276
18. Mortensen GK, Main KM, Andersson AM, Leffers H, Skakkebaek NE (2005) Determination of phthalate monoesters in human milk, consumer milk and infant formula by tandem mass spectrometry (LC-MS-MS). Anal Bioanal Chem 382:1084–1092

19. Calafat AM, Slakman AR, Silva MJ, Herbert AR, Needham LL (2004) Automated solid phase extraction and quantitative analysis of human milk for 13 phthalate metabolites. J Chromatogr B 805:49–56
20. Castle L, Gilbert J, Eklund T (1990) Migration of plasticizer from poly(vinyl chloride) milk tubing. Food Addit Contam 7:591–596
21. Petersen JH (1991) Survey of di-(2-ethylhexyl)phthalate plasticizer contamination of retail Danish milks. Food Addit Contam 8:701–705
22. Sharman M, Read WA, Castle L, Gilbert J (1994) Levels of di-(2-ethylhexyl)phthalate and total phthalate esters in milk, cream, butter and cheese. Food Addit Contam 11:375–385
23. Casajuana N, Lacorte S (2004) New methodology for the determination of phthalate esters, bisphenol A, bisphenol A diglycidyl ether, and nonylphenol in commercial whole milk samples. J Agric Food Chem 52:3702–3707
24. Sorensen LK (2006) Determination of phthalates in milk and milk products by liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 20:1135–1143
25. Clark K, Cousins IT, Mackay D, Yamada K (2003) Observed concentrations in the environment. In: Staples CA (ed) Phthalate esters, The handbook of environmental chemistry, vol 3, Anthropogenic compounds part Q. Springer, Berlin, pp 125–177
26. Page B, Lacroix G (1995) The occurrence of phthalate ester and di-2-ethylhexyl adipate plasticizers in Canadian packaging and food sampled in 1985–1989: a survey. Food Addit Contam 12:129–151
27. Feng Y, Zhua J, Sensenstein R (2005) Development of a headspace solid-phase microextraction method combined with gas chromatography mass spectrometry for the determination of phthalate esters in cow milk. Anal Chim Acta 538:41–48
28. Pfannhauser W, Leitner E, Siegl H (1995) Forschungsbericht: Phthalate in Lebensmitteln. Band 95, Beiträge Lebensmittelangelegenheiten, Veterinärverwaltung, Strahlenschutz, Toxikologie, Gentechnik; Bundesministerium für Gesundheit und Konsumentenschutz, Sektion III, Wien, Austria
29. MAFF (1996) Phthalates in food. Food surveillance information sheet, vol 83. Ministry of Agriculture Fisheries and Food, UK
30. MAFF (1998) Phthalates in infant formulae—follow-up survey. Food surveillance information sheet, vol 168. Ministry of Agriculture Fisheries and Food, UK
31. Petersen JH, Breindahl T (2000) Plasticizers in total diet samples, baby food and infant formulae. Food Addit Contam 17:133–141
32. Jarosova A (2006) Phthalic acid esters (PAEs) in the food chain. Czech J Food Sci 24:223–231
33. Saito I, Ueno E, Oshima H, Matsumoto H (2002) Levels of phthalates and adipates in processed foods and migration of di-isononyl adipate from polyvinyl chloride film into foods. Food Hyg Saf Sci (Shokuhin Eiseigaku Zasshi) 43:185–189
34. Stiftung Warentest (2005) Schmutziges Gold. Test 10: 18–23. Online available at http://www.test.de/themen/essen-trinken/test/Olivenoel-Schmutziges-Gold-1294892-1293329/
35. Peijnenburg WJGM, Struijs J (2006) Occurrence of phthalate esters in the environment of the Netherlands. Ecotoxicol Environ Saf 63:204–215
36. Greenpeace (2006) Gift im Karpfen. Risiko-Chemikalien gelangen aus der Umwelt in die Nahrungskette. FACTS_CHEMIE. Greenpeace Hamburg, Germany, 6. Online available at http://marktcheck.greenpeace.at/uploads/media/Karpfen_Belastung_Hintergrundinfo_April06.pdf
37. Leibowitz JN, Sarmiento R, Dugar SM, Ethridge MW (1995) Determination of six common phthalate plasticizers in grain neutral spirits and vodka. J AOAC Int 78:730–735
38. Yano K, Hirosawa N, Sakamoto Y, Katayama H (2002) Phthalate levels in beverages in Japan and Korea. Bull Environ Contam Toxicol 68:463–469
39. Wolheim A, Fauhl-Hassek C, Kappenstein O, Wittkowski R (2007) Analytik und Vorkommen von Weichmachern in Wein und Spirituosen. Deutscher Lebensmittelchemiker Tag, September 10–12, Erlangen, Germany
40. Del Carlo M, Pepe A, Sacchetti G, Compagnone D, Mastrocola D, Cichelli A (2008) Determination of phthalate esters in wine using solid-phase extraction and gas-chromatography—mass spectrometry. Food Chem 111:771–777

41. Bertoli M, Caussanel JL, Médina B (2004) Dosage des phthalates dans les vins boissons alcooliques. FV OIV, 1234, DGCCRF Laboratoire de Bordeaux/Talence, France
42. Di Bella G, Saitta M, Lo Curto S, Salvo F, Licandro G, Dugo G (2001) Production process contamination of citrus essential oils by plastic materials. J Agric Food Chem 49:3705–3708
43. Di Bella G, Saitta M, Pellegrino M, Salvo F, Dugo G (1999) Contamination of Italian citrus essential oils: presence of phthalate esters. J Agric Food Chem 47:1009–1012
44. Di Bella G, Saitta M, La Pera L, Alfa M, Dugo G (2004) Pesticide and plasticizer residues in bergamot essential oils from Calabria (Italy). Chemosphere 56:777–782
45. Tsumura Y, Ishimitsu S, Saito I, Sakai H, Kobayashi Y, Tonogai Y (2001) Eleven phthalate esters and di(2-ethylhexyl) adipate in one-week duplicate diet samples obtained from hospitals and their estimated daily intake. Food Addit Contam 18:449–460
46. Tsumura Y, Ishimitsu S, Kaihara A, Yoshii K, Nakamura Y, Tonogai Y (2001) Di(2-ethylhexyl) phthalate contamination of retail packed lunches caused by PVC gloves used in the preparation of foods. Food Addit Contam 18:569–579
47. Tsumura Y, Ishimitsu S, Saito I, Sakai H, Tsuchida Y, Tonogai Y (2003) Estimated daily intake of plasticizers in 1-week duplicate diet samples following regulation of DEHP-containing PVC gloves in Japan. Food Addit Contam 20:317–324
48. Aurela B, Kulmala H, Söderhjelm L (1999) Phthalates in paper and board packaging and their migration into Tenax and sugar. Food Addit Contam 16:571–577
49. BfR (2007) Di-isobutylphthalate in food-contact paper and board. Minutes of the meeting of the working group "Paper and board", July 5, 2007, Federal Institute for Risk Assessment (BfR), Berlin, Germany. Online available at http://www.bfr.bund.de/cm/230/di_isobutyphthalate_in_food_contact_paper_and_board.pdf
50. Fankhauser-Noti A, Biedermann-Brem S, Grob K (2006) PVC plasticizers/additives migrating from the gaskets of metal closures into oily food: Swiss market survey June 2005. Eur Food Res Technol 223:447–453
51. Stiftung Warentest (2007) Rückstände in Würzsoßen. Test 7: 24–27. Online available at http://www.test.de/themen/essen-trinken/test/Rueckstaende-in-Wuerzsossen-Scharf-und-gefaehrlich-1548786-1550433/
52. CVUA (2005) Übergang von Weichmachern aus der Dichtung von Twist-off-Verschlüssen auf in Gläsern verpackte Lebensmittel. Chemisches und Veterinäruntersuchungsamt (CVUA) Stuttgart, Germany. Online available at http://www.cvuas.de/pub/beitrag.asp?ID=443&subid=1&Thema_ID=3&Pdf=False&Aktuell=False
53. Fankhauser-Noti A, Grob K (2007) Blank problems in trace analysis of diethylhexyl and dibutyl phthalate: investigation of the sources, tips and tricks. Anal Chim Acta 582:353–360
54. Leung SC, Giang BY (1993) Phthalate esters as potential contaminants during GC analysis of environmental samples using electron capture and mass spectrometric detectors. Bull Environ Contam Toxicol 50:528–532
55. Wormuth M, Scheringer M, Vollenweider M, Hungerbühler K (2006) What are the sources of exposure to eight frequently used phthalic acid esters in Europeans? Risk Anal 26:803–824

Exposure to Polycyclic Aromatic Hydrocarbons: Bulky DNA Adducts and Cellular Responses

Frank Henkler, Kristin Stolpmann, and Andreas Luch

Abstract Environmental and dietary carcinogens such as polycyclic aromatic hydrocarbons (PAHs) have been intensively studied for decades. Although the genotoxicity of these compounds is well characterized (i.e., formation of bulky PAH–DNA adducts), molecular details on the DNA damage response triggered by PAHs in cells and tissues remain to be clarified. The conversion of hazardous PAHs into carcinogenic intermediates depends on enzyme-catalyzed biotransformation. Certain cytochrome P450-dependent monooxygenases (CYPs) play a pivotal role in PAH metabolism. In particular, CYP1A1 and 1B1 catalyze oxidation of PAHs toward primary epoxide species that can further be converted into multiple follow-up products, both nonenzymatically and enzymatically. Distinct functions between these major CYP enzymes have only been appreciated since transgenic animal models had been derived. Electrophilic PAH metabolites are capable of forming stable DNA adducts or to promote depurination at damaged nucleotide sites. During the following DNA replication cycle, bulky PAH–DNA adducts may be converted into mutations, thereby affecting hot spot sites in regulatory important genes such as *Ras*, *p53*, and others. Depending on the degree of DNA distortion and cell cycle progression, PAH–DNA adducts trigger nucleotide excision repair (NER) and various DNA damage responses that might include TP53-dependent apoptosis in certain cell types. In fact, cellular responses to bulky PAH–DNA damage are complex because distinct signaling branches such as ATM/ATR, NER, TP53, but also MAP kinases, interact and cooperate to determine the overall outcome to cellular injuries initiated by PAH–DNA adducts. Further, PAHs and other xenobiotics can also confer DNA damage via an alternative route of metabolic

F. Henkler, K. Stolpmann and A. Luch (✉)
Department of Product Safety, German Federal Institute for Risk Assessment (BfR), Max-Dohrn-Strasse 8-10, 10589 Berlin, Germany
e-mail: Andreas.Luch@bfr.bund.de

A. Luch (ed.), *Molecular, Clinical and Environmental Toxicology*, Experientia Supplementum 101, DOI 10.1007/978-3-7643-8340-4_5,

activation, which leads to the generation of PAH semiquinone radicals and reactive oxygen species (ROS). One-electron oxidations mediated by peroxidases or other enzymes can result in PAH radical cations that mainly form unstable DNA adducts subjected to depurination. In addition, generation of ROS can also trigger multiple cellular signaling pathways not directly related to mutagenic or cytotoxic effects, including those mediated by NFκB, SAPK/JNK, and p38. In recent years, it became clear that PAHs may also be involved in inflammatory diseases, autoimmune disorders, or atherosclerosis. Further research is under way to better characterize the significance of such newly recognized systemic effects of PAHs and to reconsider risk assessment for human health.

Keywords Apoptosis · Diol-epoxide pathway · DNA adducts · DNA repair · Growth arrest · Inflammation · Oxidative stress · PAH radical cations · Proliferation

Introduction

Polycyclic aromatic hydrocarbons (PAHs) contain two or more aromatic benzo rings fused together in a linear or angular configuration. Anthracene and phenanthrene are the simplest examples for both different types of benzo ring arrangements (Fig. 1). Parent PAHs consist of carbon and hydrogen only. However,

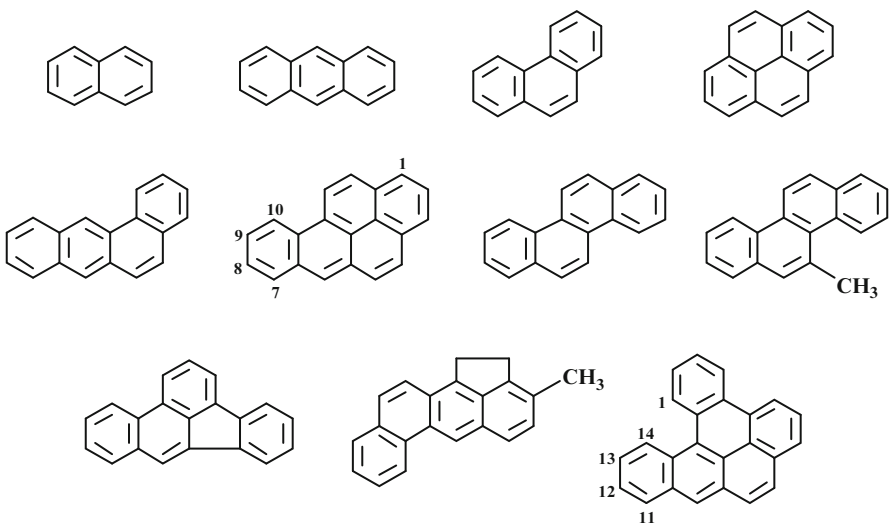


Fig. 1 Structures of different polycyclic aromatic hydrocarbons (PAHs). Compounds depicted from the *upper left side* to the *lower right side* are naphthalene, anthracene, phenanthrene, pyrene, benz[*a*]anthracene, benzo[*a*]pyrene (BP), chrysene, 5-methylchrysene, benzo[*b*]fluoranthene, 3-methylcholanthrene (3-MC), and dibenzo[*a,l*]pyrene (DBP)

the follow-up products of PAHs, formed during biotransformation, contain heteroatoms such as O or S and others (e.g., hydroxy and epoxy group-containing reactive intermediates such as diol-epoxides). Additionally, PAHs may also contain nonaromatic molecule substructures such as alkyl side chains (e.g., 5-methylchrysene), ethylene bridges (e.g., 3-methylcholanthrene, 3-MC), or pentacyclic rings (benzo[*b*]fluoranthene). For an overview of common PAH structures, please refer to Fig. 1.

Due to their aromaticity and lack of polar substituents, parent PAHs behave as lipophilic and chemically inert compounds. There are great numbers of different PAHs ("congeners") present in the environment due to their natural occurrence in crude oil and its depositories. Another important source is their generation through all kinds of incomplete combustion processes [1, 2]. PAHs usually show up as planar compounds, unless sterically demanding moieties or bridges would induce out-of-plane distortions (cf. below). Such distortions then may render molecules three-dimensional, thereby changing not only physicochemical features but also biological characteristics [3]. Many chemical species among the subgroups of tetra-, penta-, and hexacyclic PAHs, or their mixtures, have been proven genotoxic and carcinogenic in animals and—most likely—also in humans [2]. The prototypic carcinogenic PAH that serves as lead compound now since decades is benzo[*a*]pyrene (BP, Fig. 1). Although there is no unequivocal epidemiological proof for individual PAHs possible due to their general occurrence in highly complex mixtures, BP has recently been judged *carcinogenic to humans* based on a wealth of experimental data and its molecular mode of action that does not differ between cells from humans and animals [4].

Formation of DNA Adducts and Induction of DNA Damage

Upon absorption via lung, gut, and/or skin, PAHs are rapidly metabolized by cytochrome P450-dependent monooxygenases (CYPs) and peroxidases to form electrophilically reactive intermediates [2]. Two main pathways of activation have been identified. First, CYP-mediated monooxygenation of PAHs generates highly reactive epoxides that can further be hydrolyzed into dihydrodiols ("diols") and diol-epoxides. On the other hand, PAHs can also be metabolically converted into *ortho*-quinones, thereby generating reactive oxygen species (ROS) via redox cycling [2, 5]. Further, CYP- or peroxidase-catalyzed one-electron oxidation of certain carcinogenic PAHs has been shown to generate radical cations that induce DNA damage [6, 7]. While the epoxide pathway leads to the formation of stable DNA adducts, mainly at the N^2 position of 2′-deoxyguanosine (dG) or the N^6 position of 2′-deoxyadenosine (dA), the radical cations generate labile DNA adducts such as C8–dG, N7–dG, or N7–dA adducts that are eliminated via depurination, resulting in apurinic sites [6–8].

Diol-epoxide Pathway

Carcinogenic PAHs share common structural features associated with their capability to cause DNA damage and to induce tumorigenesis. These structures consist of coves, termed bay or fjord regions, that are formed between angular condensed aromatic rings (Fig. 2). Generation of mutagenic intermediates during initial biotransformation usually occurs at an adjunct aromatic ring of such a region and results in the formation of an epoxide ring. CYP1A1 or 1B1, two multifunctional monooxygenases, which are transcriptionally induced by certain xenobiotics via the aryl hydrocarbon receptor (AHR), play a pivotal role in epoxidation. Notably, several carcinogenic PAHs such as BP, dibenzo[*a,l*]pyrene (DBP), 7,12-dimethylbenz[*a*]anthracene (DMBA), or 3-methylcholanthrene (3-MC) are potent agonists of the AHR and can therefore induce their own metabolism [9, 10]. Further analysis of knockout mice revealed important physiological differences between CYP1A1 and 1B1. While CYP1A1 is strictly inducible through AHR activation, basal levels of expression of CYP1B1 were observed in several cell lines and various tissues, including mammary and adrenal glands, testes, and ovary [11–13]. CYP1B1 knockout mice were found almost completely protected against toxic effects triggered by DMBA or DBP [14, 15]. Although induction of CYP1A1 was observed in parallel, these mice developed lymphomas at reduced frequency. Further, CYP1B1

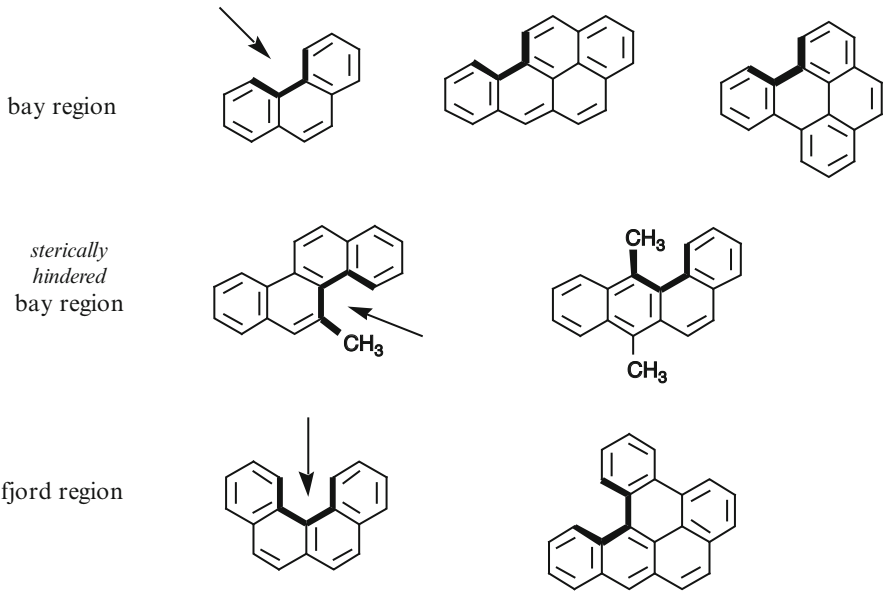


Fig. 2 The PAH bay and fjord regions: toxicologically important molecule regions of PAHs indicated by an *arrow*. Compounds depicted from the *upper left side* to the *lower right side* are phenanthrene, benzo[*a*]pyrene (BP), benzo[*e*]pyrene, 5-methylchrysene, 7,12-dimethylbenz[*a*]-anthracene (DMBA), benzo[*c*]phenanthrene (BPh), and dibenzo[*a,l*]pyrene (DBP)

triggers BP-mediated toxic effects such as wasting or immunodeficiency in mice *in vivo*, while CYP1A1 is rather involved in detoxification [16, 17]. Despite their importance for initial metabolism of PAHs toward epoxides, these observations suggest functional differences or different regulation of both enzymes *in vivo*.

The primary epoxide is then enzymatically hydrolyzed into the corresponding dihydrodiol ("diol") by microsomal epoxide hydrolase (EPHX1). At this stage, various routes of metabolism diverge. A second epoxidation can occur within the same benzo ring, thereby generating a vicinal diol-epoxide moiety (Fig. 3). This step is again mediated by CYPs, predominantly by CYP1A1, 1B1, 1A2, and 3A4. The resulting metabolites, e.g., BP 7,8-diol-9,10-epoxides (BPDEs), are highly reactive electrophilic species. Moreover, the course of CYP-mediated bioactivation usually leads to the preferential formation of a certain stereoisomer, i.e., the (*R*,*S*)-diol (*S*,*R*)-epoxide. In the case of BP, CYP- and EPHX1-mediated

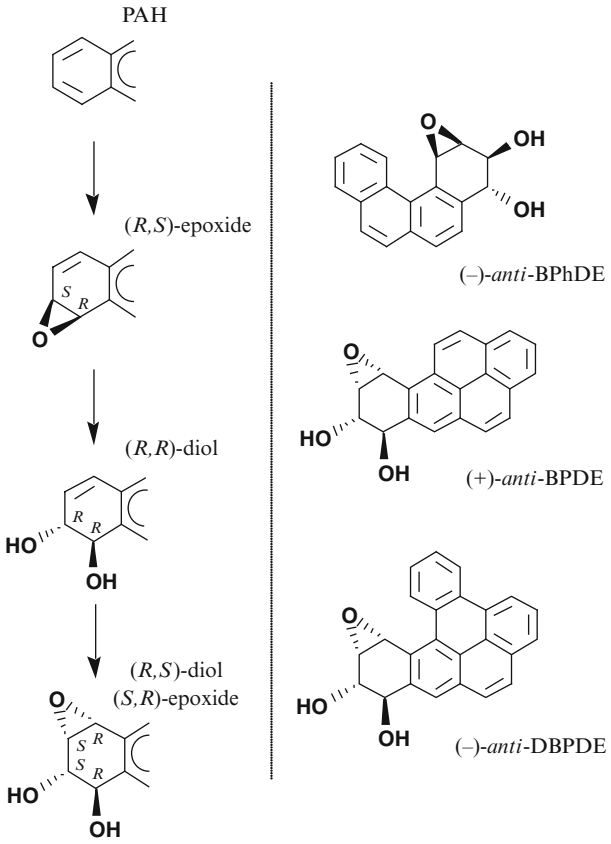


Fig. 3 Enzymatically catalyzed, preferential stereoselective biotransformation of carcinogenic PAHs toward DNA damaging (*R*,*S*)-diol (*S*,*R*)-epoxides of benzo[*c*]phenanthrene (BPhDE), benzo[*a*]pyrene (BPDE), and dibenzo[*a*,*l*]pyrene (DBPDE) (see text for further explanations)

stereoselective activation at the bay region therefore results in the formation of an excess of (+)-*anti*-BPDE (Fig. 3). Animal and *in vitro* studies also identified various dibenzopyrenes as highly potent genotoxins and carcinogens [18, 19]. However, DBP is converted by CYPs more equally into two diastereomeric 11,12-diol 13, 14-epoxides (DBPDE), the (+)-*syn*-(11*S*,12*R*,13*S*,14*R*)- and (−)-*anti*-(11*R*,12*S*, 13*S*,14*R*)-DBPDE [20–23]. These electrophilic metabolites are again capable of forming covalent reaction products with DNA, resulting in stable DBPDE–DNA adducts.

For diol-epoxide reactivity toward cellular nucleophiles (e.g., proteins, DNA), it has been postulated that the epoxide ring opens by interaction with a proton, thereby forming a transitional carbonium ion (S_N1-type of reaction). Subsequently, a covalent connection to the nucleophilic center of—for instance—the purine bases within DNA can be formed [24, 25]. A high preference of PAH diol-epoxides for exocyclic amino groups of guanine and adenine has been demonstrated both *in vitro* and *in vivo* [25–27]. In mammalian cells, DNA adducts of PAH diol-epoxides cause point mutations such as G → T or A → T transversions during the next replication cycle [25, 28]. Moreover, similar to PAH metabolism, DNA adduct formation also occurs in a stereoselective manner [2, 24]. For instance, the predominant metabolite of the bay region PAH BP, (+)-*anti*-BPDE, reacts with the exocyclic amino group of guanine, thereby forming the (+)-*trans*-*anti*-BPDE–N^2–dG adduct [2, 3, 29–31]. Conversely, fjord region PAHs typically generate N^6–dA adducts that preferentially induce A → T transversions. As well-characterized example, enzymatic metabolism of DBP was shown to lead to the generation of (−)-*trans*-*anti*-DBPDE–N^6–dA adducts [21, 23]. Although such kinds of DNA adducts promote mispairing of nucleobases, mutagenesis requires additional factors. For instance, the modified residue must escape recognition by the DNA repair machinery and, at the same time, an apoptotic DNA damage response needs to be suppressed.

Replicative polymerases blocked by DNA adducts can be replaced by enzymes of the Y-family that lack proofreading activity and thus bypass lesions in an error-prone manner [32–36]. Four Y-polymerases have been identified in humans which differ in their substrate specificity and which generate typical spectra of mutations [32, 37]. The crystal structures of the catalytical domain of polymerase κ complexed at BPDE–dA [38] or BPDE–dG adducts [39] have previously been determined, showing different conformations within DNA lesions. Adducts are either intercalated between base pairs or—alternatively—associated with the major groove. Such conformations modulate the capacity of Y-polymerases to bypass the lesion. Notably, external factors such as organic solvents have been shown to stabilize conformations that promote mutagenesis [32]. Mutagenicity also depends on the position of a modified residue and the surrounding nucleotides within the DNA sequence. Carcinogenic PAHs frequently trigger mutations at so-called hot spot sites such as certain proto-oncogenes or tumor suppressor genes that have been detected containing characteristic base pair alterations [3, 40].

Bulky DNA adducts generated by PAHs have also been shown in animal models and cultured cells to trigger mismatch mutations in proto-oncogenes, especially members of the *Ras* family (N-, H-, K-*Ras*) [3, 40]. Further, mutational patterns

found in cigarette smoke-induced lung carcinoma indicate the relevance of PAH–DNA adducts also in human carcinogenesis. Importantly, mutations within the *p53* gene, including codon 249G → T transitions, are frequently observed in human lung exposed to PAHs [41–43]. These mutations compromise the TP53-dependent tumor-suppressing activity as described below. Further, *p53* mutations were found in about 71% of female Chinese tumor patients who were constantly exposed to smoky coal in their earlier life [44], but who did not smoke tobacco. This study demonstrated specifically for a pivotal role of the *p53* gene as cellular target in PAH-induced tumorigenesis. In addition, mutations in codon 12 of the *Ras* proto-oncogene that are probably attributable to PAH exposure were also demonstrated in parallel [44]. Comparable mutation patterns also occur in tobacco-associated lung tumors. However, individual contributions of single PAHs to the overall genotoxicity and mutagenicity induced by complex PAH mixtures remain uncertain.

Alternative Toxication Routes of PAHs

Metabolism of various PAHs may lead to the generation of ROS that are capable of inducing mutations independent from the diol-epoxide pathway (Fig. 4) [2, 5, 45]. A wide range of different oxidative modifications have been identified in DNA yet. ROS represent the major endogenous source of at least 20 different DNA modifications and adducts. These species are generated either via metal-catalyzed aerobic metabolism (so-called Fenton reaction) or by phagocytic cells, antitumoral agents, and ionizing agents [46–49]. The importance of oxidative DNA damage in tumor development and neurodegenerative diseases has been demonstrated in multiple studies [46, 50, 51]. The most important endogenously formed oxidants are (in the order of decreasing reactivity) the hydroxyl radical ($HO^{\bullet}$), which interacts with biomolecules immediately after its formation via a Fenton-type reaction, the superoxide anion radical ($O_2^{-}\bullet$) that is enzymatically generated by recruitment and transfer of electrons from the mitochondrial electron transport chain or by phagocytic cells in inflamed tissue, and hydrogen peroxide (H_2O_2), which is derived from the superoxide anion radical by superoxide dismutase activity [52, 53]. H_2O_2 itself can generate $HO^{\bullet}$ in a Fenton reaction with cytoplasmic redox metals (e.g., copper, iron, Fig. 4) [5]. Upon formation, ROS can directly attack both the nucleobases and the desoxyribosyl backbone of the DNA. In addition, degradation of cell membrane lipids catalyzed by ROS may lead to certain intermediates that bind to DNA [2, 54–56]. The most intensely studied ROS-mediated DNA modification is 8-hydroxy 2′-desoxyguanosine (8-HO-dG, Fig. 4). This derivative is known to be mutagenic *in vitro* and probably also *in vivo* through causing faulty incorporation of nucleotides by DNA polymerase and inducing G → T transversions [45, 57]. Again, these transitions are being frequently found in mutated proto-oncogenes (*Myc*, *Ras*) and tumor suppressor genes (*p53, Apc*) [58, 59]. Using site-specific

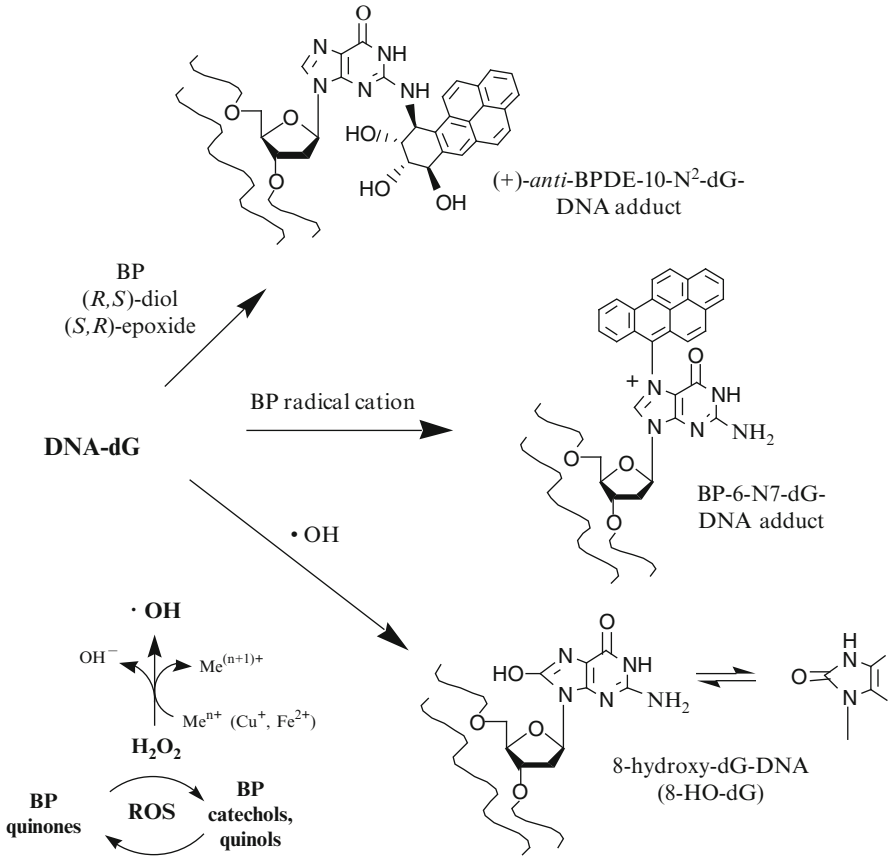


Fig. 4 Toxification routes of PAHs (e.g., benzo[*a*]pyrene, BP) and resulting DNA adducts or modifications: CYP-mediated monooxygenation route toward diol-epoxides (cf. Fig. 3), peroxidase-catalyzed one-electron oxidation toward radical cations, and nucleobase oxidation via intermediately formed hydroxy radicals (see text for further explanations)

approaches or reverse chemical mutagenesis, the mutagenic potential of oxidatively modified DNA bases has been proven already in the 1990s [60, 61].

An important mechanism of PAH-dependent ROS generation involves conversion of these compounds into quinones [2, 5, 24]. This occurs by primary conversion into phenolic intermediates that can then undergo further oxidation. For BP, 1,6-, 3,6-, and 6,12-quinones have been described already in the 1970s [62, 63]. As an alternative mechanism, formation of quinones may compete with the diol-epoxide pathway. Instead of secondary epoxidation, the earlier discussed 7,8-diol of BP can also undergo NADPH-dependent enzymatic reduction to form the corresponding catechol [2, 24]. Due to its instability, this intermediate is then further oxidized to the corresponding *ortho*-quinone. During this process, H_2O_2 and O_2^{-}• will be generated (Fig. 4). Importantly, quinones are substrates of various

reductases, such as aldo-keto reductases (AKRs) [2, 24, 45, 64]. As long as the reducing capacity of cells is maintained, these compounds can be converted back into hydroquinones (quinols) or catechols that again can undergo autoxidation, thereby generating H_2O_2 and $O_2^{-}\bullet$ in a futile redox cycling process. Similar mechanisms apply for other quinonic derivates of BP and other PAHs as well. Redox cycling is a major source for ROS in cells exposed to PAHs. Besides oxidative DNA damage, quinones are highly electrophilic compounds that may also form covalent DNA adducts via Michael-type of reactions [2, 24, 45, 64–66].

Several enzymes, in particular peroxidases, prostaglandin *H* synthase, and CYPs are suggested to initiate alternative routes of PAH metabolism by one-electron oxidation [6, 67]. Enzymatic abstraction of an electron leads to the formation of a positively charged electrophilic PAH cation radical (Fig. 4). These radicals might rapidly react with DNA to form adducts, preferentially at N3, N7, or C8 residues of purines. Due to labilization of the *N*-glycosidic bonds involved, the PAH–DNA adducts subsequently fall off the DNA sugar backbone, thereby producing apurinic sites within the sequence [6]. It has been proposed that both PAH cation radical-induced depurination [67–69] and formation of stable diol-epoxide–DNA adducts [70–72] crucially contribute to PAH-mediated carcinogenesis.

The benzylic ester pathway represents another route of PAH metabolism associated with the formation of potentially mutagenic metabolites. Based on the early observation that alkylation outside the bay region can enhance the mutagenic effects of some PAHs [73, 74], this modification was suggested to facilitate consecutive routes of metabolism such as CYP-dependent conversion into hydroxymethyl intermediates. Hydroxymethyl-PAHs may then be converted into electrophilically active benzylic esters, as for example sulfooxyalkyl intermediates [75–77]. *O*-Sulfonation is mediated by cytosolic sulfotransferases (SULT), which belong to a superfamily consisting of 11 enzymes in humans [78, 79]. The relevance of this pathway is still a matter of debate, although it was demonstrated that replacement of 1-hydroxymethylpyrene by its corresponding sulfate ester did enhance genotoxicity in rat liver [80, 81]. Importantly, some but not all derivatives of methylated PAHs, such as 6-hydroxymethyl- and 6-sulfooxymethyl-BP, have been characterized as strong carcinogens in rodents [75, 76].

DNA Damage and Mutagenic Effects

Sustainment of genome integrity and fidelity is essential for the survival of organisms. This task requires surveillance mechanisms dedicated to monitor and supervise chromosome structure and integrity and to trigger damage responses by coordinating cell cycle progression, DNA repair, and—if the damage is severe—cell death. Bulky DNA adducts would interfere with regular DNA replication and transcription and thus might induce mutations in critical genes that contribute to tumorigenesis [82–86]. During evolution, eukaryotic cells developed a versatile response system to countervail the adverse effects of such lesions. DNA damage

responses utilize distinct checkpoints to delay cell cycle progression in order to provide an opportunity for the repair of injuries [82, 83, 87]. Depending on the level of damage, this cascade can either delay a cell-destructive response or trigger activation of programmed cell death [88, 89]. For BP and other PAHs, the magnitude of DNA adduct formation depends on the metabolic capacity of target cells [90–92]. Consequently, putative cellular responses might differ between various cells and tissues. Most investigations so far have focused on liver and lung. However, recent work demonstrated a sufficient metabolic capacity even in human skin to generate mutagenic metabolites of BP and to trigger a significant formation of PAH–DNA adducts [93].

DNA Repair Mechanisms

The integrity of the genome is ensured by several DNA repair pathways, which recognize and repair distinct types of DNA damage. The type of damage induced depends on the characteristics of the genotoxic agent. Three different main but partly overlapping mechanisms do exist in eukaryotic cells: nucleotide excision repair (NER), base excision repair (BER), and mismatch repair (MMR) [94–96].

NER is the most important mechanism for the removal of bulky DNA adducts caused by PAHs (Fig. 5), aflatoxin B_1, or UV radiation [94]. NER is partly controlled by the tumor suppressor protein TP53 and characterized by a defined substrate specificity. There are two branches of the NER pathway: global genome (GG) and transcription-coupled repair (TCR). The transcription-independent GG-NER pathway eliminates modifications from nontranscribed strands as well as from noncoding regions after a genome-wide screening and recognition of the damage by a heterodimeric XPC complex [96, 97]. This complex recognizes structural distortions within DNA helices. Both strands are then dissociated by the XPB and XPD helicases of transcription factor TFIIH. A fragment of about 20 nucleobases that contains the damaged residue is then cleaved from the injured DNA strand by endonucleases XPG and XPF–ERCC1. Finally, the segment is resynthesized by DNA polymerases δ and ε and religated by DNA ligase 1 [83, 97–99]. The efficiency of NER seems to be determined by the stereochemical adduct configuration and the base sequence context [100–104]. Analyses employing an oligonucleotide excision assay showed a highly variable efficiency for different configurations of BPDE–N^2–dG adducts [100, 101]. In contrast to GG-NER, the TCR pathway lacks a specific sensing system. Therefore, TCR can only recognize lesions that stall RNA polymerase II at the transcribed strand [96, 97, 105]. Blockage of RNA polymerase II leads to the recruitment of CSA and CSB proteins, which activate endonucleases XPG and XPF–ERCC1. Subsequent stages are then identical to GG-NER [83, 106]. Notably, there are differences in the activation of the GG-NER machinery in response to UV-induced DNA damage or bulky adducts. UV-induced DNA lesions such as cyclobutane pyrimidine dimers or 6-4 photoproducts interact with DNA damage-binding proteins (DDB) 1 and 2 that promote recruitment of the

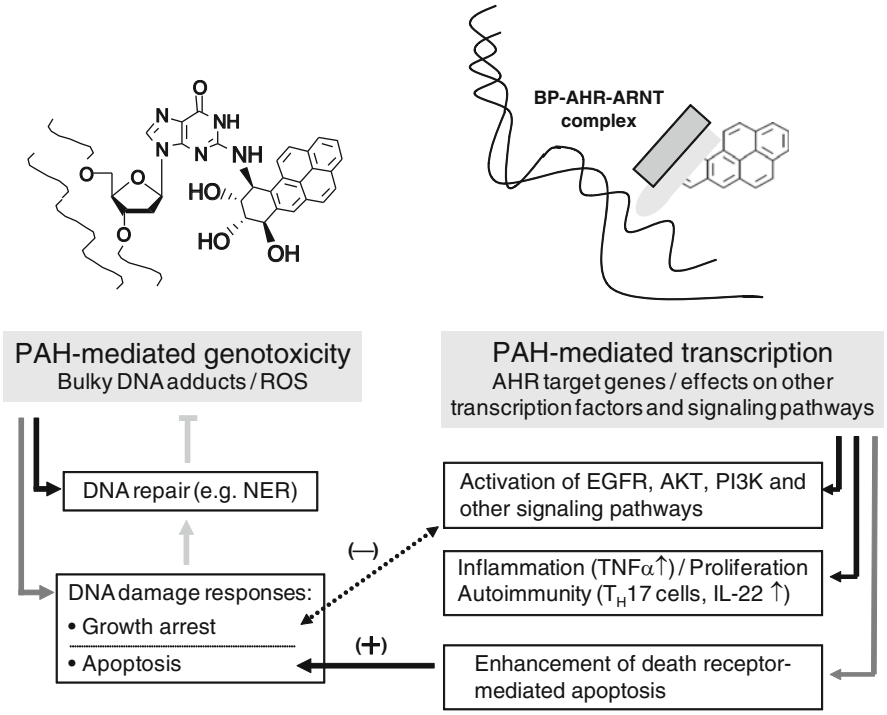


Fig. 5 PAH–DNA damage responses and PAH-mediated effects independent from DNA adduct formation (see text for further explanations). *AHR* aryl hydrocarbon receptor, *AKT* protein kinase B, *ARNT* AHR nuclear translocator, *BP* benzo[*a*]pyrene, *EGFR* EGF receptor, *NER* nucleotide excision repair, *PI3K* phosphoinositide 3-kinase, *ROS* reactive oxygen species

XPC complex and enhance excision of the injured residue. Possibly, this mechanism facilitates a more efficient NER response to UV exposure than to bulky DNA adducts, as the latter pathway seem to depend solely on the sensing by XPC and subsequent lesion tracking by TFIIH [96, 97, 107, 108].

Nucleotides that are injured due to deamination, alkylation, or ROS-mediated oxidation can be eliminated via BER [109]. Upon recognizing small structural alterations of nucleobases, DNA glycosylases eliminate damaged bases from the deoxyribosyl backbone of the DNA by hydrolysis of the *N*-glycosidic bond, thus forming a nick ("abasic site") in the helix [110]. Eleven glycosylases are known to date, with a restricted but partially overlapping specificity for DNA lesions. An accrued abasic site serves as toehold for the APE1 endonuclease, which incises the strand at the abasic site. Subsequently, lyases remove the baseless sugar residue and DNA polymerase β replaces the nucleotide. Finally, an XPCC1/DNA ligase 3 complex religates the DNA nick [111, 112].

To a minor extent, chemical-induced DNA adducts are removed by MMR. MMR proteins bind and process DNA adducts originating from compounds such

as BPDE or cisplatin and from oxidative damage [113–115]. Lesion-bound heterodimeric complexes of MutSα (or MutSβ) recruit MutLα. The trimeric complex then undergoes an ATP-driven conformational change, leading to formation and migration of a sliding clamp. After dissociation of MutSα/β from the DNA, exonuclease 1 removes the defect strand and DNA polymerase δ resynthesizes the excised segment [116]. Furthermore, MMR proteins also bind to persistent DNA adducts and affect DNA damage response pathways. So, it was shown that interactions of MMR proteins with DNA adducts trigger an apoptotic response via TP53- and TP73-dependent pathways [113, 117].

Although it is evident that DNA adducts activate DNA repair mechanisms, it is not well understood how these lesions trigger cell cycle arrest or apoptosis in injured cells. Notably, mouse keratinocytes, deficient in TCR, were protected from apoptosis when the GG-NER repair response was selectively activated by radiation [118]. Although BP was shown to induce TP53 expression [119, 120], DNA repair, and TP53-dependent apoptosis [121], it is not clear whether recognition of DNA lesions by XPC alone is sufficient to initiate DNA damage responses. The question of how PAH–DNA adducts activate cellular responses still requires exploration in much greater detail.

PAH–DNA Damage Response: Growth Arrest, Repair, or Apoptosis?

So far, most studies on PAH-induced adversity were focused on metabolism, mutagenic effects, or carcinogenesis, whereas other cellular responses triggered by PAH–DNA adducts, oxidative DNA damage, and—in particular—AHR activation are only poorly understood [10, 122]. Intriguingly, mechanisms that regulate DNA repair are closely connected with pathways that trigger adapted cellular effects, such as growth arrest or apoptosis (Fig. 5). This is illustrated by the fact that mutations within the ataxia telangiectasia mutated (ATM) and ATM- and Rad-3-related (ATR) kinases do not only affect DNA repair, but inhibit an apoptotic response to genotoxic stress or radiation as well [123–125]. In growth-arrested cells, the G1 → S phase transition is prevented and DNA repair mechanisms can proceed, whereas the alternative apoptotic response is an irreversible process that eliminates injured cells. The interactions between DNA damage response, cell cycle checkpoints, and apoptosis are very complex [105, 126]. Again, members of the ATM/ATR kinase family play a crucial role in DNA damage response. In humans, both ATM and ATR kinases accumulate rapidly at DNA lesions, become phosphorylated, and provide a scaffold to recruit DNA repair enzymes, accessory proteins, and factors that regulate the overall cellular responses. It is not precisely known how DNA lesions are recognized by the ATM/ATR kinases, and it is possible that these enzymes also play additional roles in the recognition of injured loci. In fact, DNA-dependent protein kinase (DNA-PK), which does also belong to

the ATM/ATR family [127], mediates a combined function in recognition and repair of double-strand breaks, too.

Activated ATM and ATR trigger multiple cellular responses [128]. Firstly, these kinases are implicated to inhibit G1 → S transition rapidly after exposure of replicating cells to UV radiation. This checkpoint involves activation of CHK1, CHK2, and CDC25, leading to an inhibitory phosphorylation of CDK2. So far, it is not known whether this mechanism is also activated by excessive exposure to chemical mutagens, such as PAHs. Phosphorylation and stabilization of the tumor suppressor protein TP53 is then required to sustain cell cycle arrest. This involves transcriptional upregulation of cyclin-dependent kinase inhibitors, such as $p21^{CIP/WAF1}$. BP was earlier shown to trigger TP53-dependent G1 arrest in fibroblasts [129]. Intriguingly, redundant mechanisms have been described that trigger cell cycle arrest also at later stages, either during S phase [130, 131] or at the G2 → M transition [132]. These TP53-independent pathways did however require ATM/ATR, as well as CHK1 signaling. It is much likely that the cellular responses to PAH–DNA adducts may strongly depend on the cell type or on parameters of exposure that relate to particular mutagens.

Notably, besides growth arrest in G1, TP53 simultaneously promotes DNA repair, in particular GG-NER [133]. However, TP53-mediated transcriptional upregulation of BH3-only proteins, such as PUMA, NOXA, or BAX, can also shift the cellular response toward apoptosis. Again, the regulation is very complex, as TP53 induces both proapoptotic proteins and inhibitors of apoptosis, such as BCL2. On the other hand, XPC—a pivotal component of the NER machinery (cf. above)—is also a target gene of TP53 [134, 135]. A role of TP53 in the repair of PAH–DNA adducts via GG-NER was previously shown [136–138]. Exposures to BP or other PAHs led further to induction of TP53 and to transcription of TP53-dependent target genes [119, 120, 139–141]. Although bulky PAH–DNA adducts can trigger TP53-dependent DNA damage responses, the precise molecular regulation remain enigmatic. Components of the NER machinery are required for cell cycle checkpoint activation, but it is still unclear whether GG-NER alone is sufficient to activate TP53 and/or other factors that regulate TP53-independent growth arrest.

An interesting link between cell cycle phase and activation of GG-NER and ATR kinase has recently been demonstrated by Auclair and colleagues [142, 143]. In these experiments, removal of cyclobutane pyrimidine dimers by GG-NER was strictly dependent on ATR during S phase, but not at other stages of the cell cycle. Further, DNA adducts formed during cisplatin treatment were shown to trigger phosphorylation of ATM, which interacted directly with the XPC complex that becomes recruited to damaged DNA loci (cf. above). Another direct interaction between ATM and the basal transcription factor TFIIH has been found to be mediated by XPG [144]. In addition, ATR activates components of the NER machinery, such as XPA, [145] and promotes its nuclear import [146]. These data point to a coordinated regulation of GG-NER and ATM-/ATR-induced responses to DNA adducts—a cross talk that was shown to facilitate DNA repair and survival of injured cells [147].

On the other hand, there is only limited evidence that PAH-mediated genotoxicity alone is sufficient to induce apoptosis. Although excessive exposure of fibroblasts to carcinogenic PAH metabolites was shown to trigger TP53-dependent cytotoxicity [121] and BP was demonstrated to cause apoptosis in liver cells [148, 149], the overall proapoptotic capacity of PAH–DNA adducts remains elusive and might depend on cell type and environment. The factors that ultimately switch the TP53 response from growth arrest and repair to apoptosis are not well defined. With respect to BP, an important role in this cellular decision making has been assigned to the p38 MAPK by several reports [150, 151]. p38 MAPK is involved in cellular signaling triggered by various stress stimuli. For example, this kinase phosphorylates TP53 in response to UV irradiation and enhances its apoptotic effects [152, 153]. The interplay between BP exposure and p38 activation remains to be clarified in detail. Furthermore, proapoptotic effects of BP that have been observed in several cell lines might also be triggered by other, TP53-independent pathways.

PAH–DNA adducts can persist into S phase, for instance, due to failing adduct recognition and checkpoint initiation, or insufficient damage repair as frequently observed in TP53-deficient cells [136, 154, 155]. As a result replication stress, including blockage and disassembly of the replication fork, might occur. It has been shown that during S phase, BP-induced adducts stalled the replication forks and inhibited DNA synthesis [156]. Again, consequences might be very complex. Firstly, ATR triggers CHK1-mediated growth arrest that does not depend on TP53 and facilitates DNA repair and survival. Interestingly, GG-NER is probably not sufficient to restore BP-induced lesions, as translesional synthesis by the error-prone DNA polymerase κ is necessary in exposed fibroblasts to recover from the S phase checkpoint arrest and to resume cell cycle progression [131]. However, formation of extended single-stranded DNA regions and collapsing replication forks could trigger double-strand breaks and subsequent apoptotic responses, depending on DNA-PK and TP53. Further, a novel ATM/ATR-dependent pathway has recently been identified that links replication stress to the activation of caspases. This signaling route bypasses TP53 and the classical intrinsic apoptotic pathway, but is normally inhibited by CHK1 in injured cells [157].

A detailed model of the PAH-induced DNA damage response signaling pathways still needs to be established to describe the balance between repair/checkpoint control and proapoptotic effects. As p38 MAPK was shown to play a central role in BP-mediated cytotoxicity, it is tempting to speculate that this enzyme integrates various apoptotic and necrotic stimuli into an overall response. This hypothesis is supported by the observation that several cytotoxic stimuli or agents, including retinoids, cisplatin, and other chemotherapeutic drugs depend on activation of p38 MAPK [158]. p38 MAPK can further be activated by TP53 and TP53-independently via the proto-oncogene *c-ABL* [159]. Similar to other mitogen-activated protein kinases, p38 MAPK is also regulated by multiple extracellular stimuli that could therefore affect the overall outcome of the PAH-induced DNA damage response. Further, alternative routes of metabolism could modulate cellular responses to PAH exposure as well. For example, BP was demonstrated to promote

apoptosis via an H_2O_2-dependent mechanism that led to the activation of SAPK/JNK, Na^+/H^+ exchanger, and hexokinase II [160–162]. The authors also have shown that this pathway cooperates with TP53-dependent signaling in the execution of apoptosis in liver epithelial cells.

Effects of PAHs on Proliferation and Inflammatory Cellular Signaling

Cellular signaling cascades that are triggered by carcinogenic PAHs can affect whether genotoxically injured cells eventually survive and—as a consequence—would accumulate mutations over their life span (Fig. 5). Mitogenic effects of BP and its metabolites have been observed in human mammary epithelial cells [163], including activation of AKT (protein kinase B) [164], phosphoinositide 3-kinase (PI3K) [165], phosphorylation of SRC-homology and collagen protein (SHC), and ligand-independent activation of the EGF receptor [166]. The capacity of BP to enhance proliferation was related to conversion into quinones that led to the generation of ROS (cf. above). Both the 1,6- and 3,6-quinone of BP were shown to activate phospholipase Cγ (PLCγ) as well as several STAT proteins [166]. In concert with the mutagenic capacity, such EGF receptor-like effects of BP are likely to promote proliferation, angiogenesis, invasion, metastasis, and inhibition of apoptosis in human mammary [163] and bronchial epithelial cells [167]. As H_2O_2 and $O_2^{-}\bullet$ play a central part in signal transduction [168], it appears feasible that those PAH metabolites associated with ROS formation can directly affect multiple cellular functions, such as tyrosine phosphorylation and activation of MAP kinases (MAPKs). BP was further shown to trigger expression of proinflammatory cytokines, such as tumor necrosis factor (TNF) α. In macrophages, this effect was not inhibited by AHR antagonists and did possibly depend on ROS-mediated ERK activation [169]. In addition, TNFα was shown to modulate the biological effects of PAHs, as it did interfere with AHR signaling in hepatocytes, either enhancing the genotoxic effects of BP or even promoting proliferation at lower concentrations [170–172].

A distinction between direct signaling effects of PAH metabolites and indirect transcriptional mechanisms is often difficult to prove. Besides its function in the metabolism of xenobiotics, the AHR was also shown to regulate important cellular processes, such as proliferation, embryonic development, as well as cell adhesion and migration [10, 173]. The question of whether these functions are also affected by chronic PAH exposures still needs to be clarified. However, there is growing evidence that exposure to PAHs constitutes additional risks to human health, which are not directly related to PAH–DNA adduct formation and mutagenesis. For example, PAHs were demonstrated to induce inflammatory atherosclerosis [174] and to enhance allergic inflammation [175], respectively. Recently, AHR ligands have also been suggested to act as cofactors in the development of autoimmune

diseases [176]. The authors demonstrated that the cytokine (e.g., IL-22) expressing activity of T_H17 cells was regulated by AHR agonists. Among $CD4^+$ cells, T_H17 cells are the only subpopulation that express this receptor. AHR signaling was further shown to increase the population of T_H17 cells and to aggravate the pathology of an experimentally induced autoimmune encephalomyelitis model in mice. In addition, most recently, we have shown that AHR activation in human skin cells can also enhance the proapototic effects of CD95L (ligand of APO-1/FAS) and TNF-related apoptosis-inducing ligand (TRAIL) [177] and that these effects were neither dependent on induction of CYP1A1/1B1 nor on the generation of genotoxic metabolites or formation of PAH–DNA adducts. All of these data point to the intriguing possibility that chronic environmental exposures to PAHs might be linked to the etiology of emerging diseases, such as allergies or certain autoimmune disorders, or affect regulation of apotosis in inflammatory skin diseases.

Summary

Formation of bulky DNA adducts is regarded as pivotal carcinogenic risk factor associated with certain high molecular weight PAHs that share structural features termed bay and fjord regions. Certain PAH–DNA adducts are known to trigger mutations in oncogenes or tumor suppressor genes in animal models but also in humans who had been highly exposed to PAHs. Besides DNA repair, cellular responses also include activation of TP53-dependent growth arrest and apoptosis.

The mutagenic diol-epoxide pathway depends on AHR-mediated induction of the activating enzymes such as CYP1A1 and 1B1. Certain mutagenic PAHs that are acting as strong AHR agonists thus promote their own metabolic activation, subsequently leading to genotoxic effects such as DNA adduct formation and/or oxidative damage. Besides the long-established role of AHR in CYP-mediated metabolism of xenobiotics, a wider functional spectrum and physiological relevance have been recognized in recent years. This includes multiple novel functions in development, inflammation, and autoimmunity that are triggered by both endogenous and exogenous ligands. However, modulating effects of xenobiotic agonists, as well as consequences for the overall toxicity of PAHs and additional adverse effects, remain to be investigated in much greater detail in the years ahead.

References

1. Harvey RG (1991) Polycyclic aromatic hydrocarbons: chemistry and carcinogenicity. Cambridge University Press, Cambridge
2. Luch A, Baird WM (2010) Carcinogenic polycyclic aromatic hydrocarbons. In: McQueen CA (ed) Comprehensive toxicology, vol 14, 2nd edn. Academic, Oxford, pp 85–123
3. Luch A (2009) On the impact of the molecule structure in chemical carcinogenesis. In: Luch A (ed) Molecular, clinical and environmental toxicology, vol 1. Birkhäuser, Basel, pp 151–179

4. Cogliano VJ, Baan RA, Straif K, Grosse Y, Secretan B, El Ghissassi F (2008) Use of mechanistic data in IARC evaluations. Environ Mol Mutagen 49:100–109
5. Goetz ME, Luch A (2008) Reactive species: a cell damaging rout assisting to chemical carcinogens. Cancer Lett 266:73–83
6. Cavalieri EL, Rogan EG (1995) Central role of radical cations in metabolic activation of polycyclic aromatic hydrocarbons. Xenobiotica 25:677–688
7. Melendez-Colon VJ, Luch A, Seidel A, Baird WM (2000) Formation of stable DNA adducts and apurinic sites upon metabolic activation of bay and fjord region polycyclic aromatic hydrocarbons in human cell cultures. Chem Res Toxicol 13:10–17
8. RamaKrishna NV, Gao F, Padmavathi NS, Cavalieri EL, Rogan EG, Cerny RL, Gross ML (1992) Model adducts of benzo[*a*]pyrene and nucleosides formed from its radical cation and diol epoxide. Chem Res Toxicol 5:293–302
9. Beischlag TV, Luis Morales J, Hollingshead BD, Perdew GH (2008) The aryl hydrocarbon receptor complex and the control of gene expression. Crit Rev Eukaryot Gene Expr 18:207–250
10. Puga A, Ma C, Marlowe JL (2009) The aryl hydrocarbon receptor cross-talks with multiple signal transduction pathways. Biochem Pharmacol 77:713–722
11. Ding X, Kaminsky LS (2003) Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. Annu Rev Pharmacol Toxicol 43:149–173
12. Nebert DW, Dalton TP (2006) The role of cytochrome P450 enzymes in endogenous signalling pathways and environmental carcinogenesis. Nat Rev Cancer 6:947–960
13. Taylor RT, Wang F, Hsu EL, Hankinson O (2009) Roles of coactivator proteins in dioxin induction of CYP1A1 and CYP1B1 in human breast cancer cells. Toxicol Sci 107:1–8
14. Buters JT, Sakai S, Richter T, Pineau T, Alexander DL, Savas U, Doehmer J, Ward JM, Jefcoate CR, Gonzalez FJ (1999) Cytochrome P450 CYP1B1 determines susceptibility to 7,12-dimethylbenz[*a*]anthracene-induced lymphomas. Proc Natl Acad Sci USA 96: 1977–1982
15. Buters JT, Mahadevan B, Quintanilla-Martinez L, Gonzalez FJ, Greim H, Baird WM, Luch A (2002) Cytochrome P450 1B1 determines susceptibility to dibenzo[*a,l*]pyrene-induced tumor formation. Chem Res Toxicol 15:1127–1135
16. Uno S, Dalton TP, Derkenne S, Curran CP, Miller ML, Shertzer HG, Nebert DW (2004) Oral exposure to benzo[*a*]pyrene in the mouse: detoxication by inducible cytochrome P450 is more important than metabolic activation. Mol Pharmacol 65:1225–1237
17. Uno S, Dalton TP, Dragin N, Curran CP, Derkenne S, Miller ML, Shertzer HG, Gonzalez FJ, Nebert DW (2006) Oral benzo[*a*]pyrene in Cyp1 knockout mouse lines: CYP1A1 important in detoxication, CYP1B1 metabolism required for immune damage independent of total-body burden and clearance rate. Mol Pharmacol 69:1103–1114
18. Chang R, Levin W, Wood AW, Lehr RE, Kumar S, Yagi H, Yerina DM, Conney AH (1982) Tumorigenicity of bay-region diol-epoxides and other benzo-ring derivatives of dibenzo[*a,h*] pyrene and dibenzo[*a, i*]pyrene on mouse skin and in newborn mice. Cancer Res 42:25–29
19. Busby WF Jr, Smith H, Crespi CL, Penman BW (1995) Mutagenicity of benzo[*a*]pyrene and dibenzopyrenes in the *Salmonella typhimurium* TM677 and the MCL-5 human cell forward mutation assays. Mutat Res 342:9–16
20. Luch A, Seidel A, Glatt H, Platt KL (1997) Metabolic activation of the (+)-*S*,*S*- and (–)-*R*, *R*-enantiomers of trans-11,12-dihydroxy-11,12-dihydrodibenzo[a, l]pyrene: stereoselectivity, DNA adduct formation, and mutagenicity in Chinese hamster V79 cells. Chem Res Toxicol 10: 1161–1170
21. Luch A, Coffing SL, Tang YM, Schneider A, Soballa V, Greim H, Jefcoate CR, Seidel A, Greenlee WF, Baird WM, Doehmer J (1998) Stable expression of human cytochrome P450 1B1 in V79 Chinese hamster cells and metabolically catalyzed DNA adduct formation of dibenzo[*a,l*]pyrene. Chem Res Toxicol 11:686–695
22. Luch A, Schober W, Soballa VJ, Raab G, Greim H, Jacob J, Doehmer J, Seidel A (1999) Metabolic activation of dibenzo[*a,l*]pyrene by human cytochrome P450 1A1 and P450 1B1 expressed in V79 Chinese hamster cells. Chem Res Toxicol 12:353–364

23. Schober W, Luch A, Soballa VJ, Raab G, Stegemann JJ, Doehmer J, Jacob J, Seidel A (2006) On the species-specific biotransformation of dibenzo[*a,l*]pyrene. Chem Biol Interact 161: 37–48
24. Luch A, Baird WM (2005) Metabolic activation and detoxification of polycyclic aromatic hydrocarbons. In: Luch A (ed) The carcinogenic effects of polycyclic aromatic hydrocarbons. Imperial College Press, London, pp 19–95
25. Luch A (2005) Polycyclic aromatic hydrocarbon-induced carcinogenesis—An integrated view. In: Luch A (ed) The carcinogenic effects of polycyclic aromatic hydrocarbons. Imperial College Press, London, pp 381–454
26. Jerina DM, Chadha A, Cheh AM, Schurdak ME, Wood AW, Sayer JM (1991) Covalent bonding of bay-region diol epoxides to nucleic acids. Adv Exp Med Biol 283:533–553
27. Geacintov NE, Cosman M, Hingerty BE, Amin S, Broyde S, Patel DJ (1997) NMR solution structures of stereomeric polycyclic aromatic carcinogen-DNA adducts: principles, patterns and diversity. Chem Res Toxicol 10:111–146
28. Dipple A, Khan QA, Page JE, Pontén I, Szeliga J (1999) DNA reactions, mutagenic action and stealth properties of polycyclic aromatic hydrocarbon carcinogens. Int J Oncol 14: 103–111
29. Koreeda M, Moore PD, Wislocki PG, Levin W, Yagi H, Jerina DM (1978) Binding of benzo[*a*]pyrene 7,8-diol-9,10-epoxides to DNA, RNA, and protein of mouse skin occurs with high stereoselectivity. Science 199:778–781
30. Dipple A (1994) Reactions of polycyclic aromatic hydrocarbons with DNA. In: Hemminki K, Dipple A, Shuker DEG, Kadlubar FF, Segerbäck D, Bartsch H (eds) DNA adducts: identification and biological significance, vol 125. International Agency for Research on Cancer, IARC Sci Publications, Lyon, France, pp 107–129
31. Szeliga J, Dipple A (1998) DNA adduct formation by polycyclic aromatic hydrocarbon dihydrodiol epoxides. Chem Res Toxicol 11:1–11
32. Yang W (2005) Portraits of a Y-family DNA polymerase. FEBS Lett 579:868–872
33. Ohmori H, Friedberg EC, Fuchs RP, Goodman MF, Hanaoka F, Hinkle D, Kunkel TA, Lawrence CW, Livneh Z, Nohmi T, Prakash L, Todo T, Walker GC, Wang Z, Woodgate R (2001) The Y-family of DNA polymerases. Mol Cell 8:7–8
34. Sweasy JB, Lauper JM, Eckert KA (2006) DNA polymerases and human diseases. Radiat Res 166:693–714
35. Guo C, Kosarek-Stancel JN, Tang TS, Friedberg EC (2009) Y-family of DNA polymerases in mammalian cells. Cell Mol Life Sci 66:2363–2381
36. Cruet-Henneguart S, Gallagher K, Sokòl AM, Villalan S, Prendergast AM, Carty MP (2010) DNA polymerase η, a key protein in translesion synthesis in human cells. Subcell Biochem 50:189–209
37. Broyde S, Wang L, Rechkoblit O, Geacintov NE, Patel DJ (2008) Lesion processing: high-fidelity *versus* lesion-bypass DNA polymerases. Trends Biochem Sci 33:209–219
38. Ling H, Sayer JM, Plosky BS, Yagi H, Boudsocq F, Woodgate R, Jerina DM, Yang W (2004) Crystal structure of a benzo[*a*]pyrene diol epoxide adduct in a ternary complex with a DNA polymerase. Proc Natl Acad Sci USA 101:2265–2269
39. Bauer J, Xing G, Yagi H, Sayer JM, Jerina DM, Ling H (2007) A structural gap in Dpo4 supports mutagenic bypass of a major benzo[*a*]pyrene dG adduct in DNA through template misalignment. Proc Natl Acad Sci USA 104:14905–14910
40. Sills RC, Boorman GA, Neal JE, Hong HL, Devereux TR (1999) Mutations in *ras* genes in experimental tumours of rodents. IARC Sci Publ 146:55–86
41. Denissenko MF, Pao A, Tang M, Pfeifer GP (1996) Preferential formation of benzo[*a*]pyrene adducts at lung cancer mutational hotspots in *p53*. Science 274:430–432
42. Denissenko MF, Chen JX, Tang MS, Pfeifer GP (1997) Cytosine methylation determines hot spots of DNA damage in the human *p53* gene. Proc Natl Acad Sci USA 94:3893–3898
43. Hussain SP, Amstad P, Raja K, Sawyer M, Hofseth L, Shields PG, Hewer A, Phillips DH, Ryberg D, Haugen A, Harris CC (2001) Mutability of *p53* hotspot codons to benzo[*a*]pyrene

diol epoxide (BPDE) and the frequency of *p53* mutations in nontumorous human lung. Cancer Res 61:6350–6355
44. DeMarini DM, Landi S, Tian D, Hanley NM, Li X, Hu F, Roop BC, Mass MJ, Keohavong P, Gao W, Olivier M, Hainaut P, Mumford JL (2001) Lung tumor *KRAS* and *TP53* mutations in nonsmokers reflect exposure to PAH-rich coal combustion emissions. Cancer Res 61:6679–6681
45. Park JH, Troxel AB, Harvey RG, Penning TM (2006) Polycyclic aromatic hydrocarbon (PAH) *o*-quinones produced by the aldo-keto-reductases (AKRs) generate abasic sites, oxidized pyrimidines, and 8-oxo-dGua via reactive oxygen species. Chem Res Toxicol 19:719–728
46. Ames BN (1989) Endogenous DNA damage as related to cancer and aging. Mutat Res 214:41–46
47. Esterbauer H, Eckl P, Ortner A (1990) Possible mutagens derived from lipids and lipid precursors. Mutat Res Rev Genet Toxicol 238:223–233
48. Lovell MA, Markesbery WR (2007) Oxidative DNA damage in mild cognitive impairment and early Alzheimer's disease. J Neurosci Res 85:3036–3040
49. Dizdaroglu M, Jaruga P, Birincioglu M, Rodriguez H (2002) Free radical-induced damage to DNA: mechanisms and measurement. Free Radic Biol Med 32:1102–1115
50. Wiseman H, Halliwell B (1996) Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. Biochem J 313:17–29
51. Lovell MA, Markesbery WR (2007) Oxidative DNA damage in mild cognitive impairment and late-stage Alzheimer's disease. Nucleic Acids Res 35:7497–7504
52. Marnett LJ (2000) Oxyradicals and DNA damage. Carcinogenesis 21:361–370
53. Wang Y (2008) Bulky DNA lesions induced by reactive oxygen species. Chem Res Toxicol 21:276–281
54. Richter C (1992) Reactive oxygen and DNA damage in mitochondria. Mutat Res 275:249–255
55. Penning TM, Burczynski ME, Hung CF, McCoull K, Palackal NT, Tsuruda LS (1999) Dihydrodiol dehydrogenases and polycyclic aromatic hydrocarbon activation: generation of reactive and redox active *o*-quinones. Chem Res Toxicol 12:1–18
56. Breen AP, Murphy JA (1995) Reactions of oxyl radicals with DNA. Free Radic Biol Med 18:1033–1077
57. Shibutani S, Takeshita M, Grollman AP (1991) Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. Nature 349:431–434
58. Hussain SP, Harris CC (1998) Molecular epidemiology of human cancer: contribution of mutation spectra studies of tumor suppressor genes. Cancer Res 58:4023–4037
59. Ambs S, Bennett WP, Merriam WG, Ogunfusika MO, Oser SM, Harrington AM, Shields PG, Felley-Bosco E, Hussain SP, Harris CC (1999) Relationship between *p53* mutations and inducible nitric oxide synthase expression in human colorectal cancer. J Natl Cancer Inst 91:86–88
60. Feig DI, Sowers LC, Loeb LA (1994) Reverse chemical mutagenesis: identification of the mutagenic lesion resulting from reactive oxygen species-mediated damage to DNA. Proc Natl Acad Sci USA 91:6609–6613
61. Wang D, Kreutzer DA, Essigmann JM (1998) Mutagenicity and repair of oxidative DNA damage: insights from studies using defined lesions. Mutat Res 400:99–115
62. Lorentzen RJ, Caspary WJ, Lesko SA, Ts'o PO (1975) The autooxidation of 6-hydroxy-benzo[*a*]pyrene and 6-oxobenzo[*a*]pyrene radical, reactive metabolites of benzo[*a*]pyrene. Biochemistry 14:3970–3977
63. Lorentzen RJ, Ts'o PO (1977) Benzo[*a*]yrenedione/benzo[*a*]pyrenediol oxidation-reduction couples and the generation of reactive reduced molecular oxygen. Biochemistry 16: 1467–1473
64. Jin Y, Penning TM (2007) Aldo-keto reductases and bioactivation/detoxication. Annu Rev Pharmacol Toxicol 47:263–296

65. Penning TM (1993) Dihydrodiol dehydrogenase and its role in polycyclic aromatic hydrocarbon metabolism. Chem Biol Interact 89:1–34
66. Burczynski ME, Lin HK, Penning TM (1999) Isoform-specific induction of a human aldo-keto reductase by polycyclic aromatic hydrocarbons (PAHs), electrophiles, and oxidative stress: implications for the alternative pathway of PAH activation catalyzed by human dihydrodiol dehydrogenase. Cancer Res 59:607–614
67. Cavalieri EL, Rogan EG (1992) The approach to understanding aromatic hydrocarbon carcinogenesis. The central role of radical cations in metabolic activation. Pharmacol Ther 55:183–199
68. Cavalieri EL, Rogan EG (1996) The primary role of apurinic sites in tumor initiation. Polycycl Aromat Compds 10:251–258
69. Chakravarti D, Venugopal D, Mailander PC, Meza JL, Higginbotham S, Cavalieri EL, Rogan EG (2008) The role of polycyclic aromatic hydrocarbon-DNA adducts in inducing mutations in mouse skin. Mutat Res 649:161–178
70. Melendez-Colon VJ, Smith CA, Seidel A, Luch A, Platt KL, Baird WM (1997) Formation of stable adducts and absence of depurinating DNA adducts in cells and DNA treated with the potent carcinogen dibenzo[*a,l*]pyrene or its diol epoxides. Proc Natl Acad Sci USA 94: 13542–13547
71. Melendez-Colon VJ, Luch A, Seidel A, Baird WM (1999) Comparison of cytochrome P450- and peroxidase-dependent metabolic activation of the potent carcinogen dibenzo[*a,l*]pyrene in human cell lines: formation of stable DNA adducts and absence of a detectable increase in apurinic sites. Cancer Res 59:1412–1416
72. Melendez-Colon VJ, Luch A, Seidel A, Baird WM (1999) Cancer initiation by polycyclic aromatic hydrocarbons results from formation of stable DNA adducts rather than apurinic sites. Carcinogenesis 20:1885–1891
73. Hecht SS, Amin S, Melikian AA, Lavoie EJ, Hoffmann D (1985) Effects of methyl and fluorine substitutions on the metabolic activation and tumorigenicity of polycyclic aromatic hydrocarbons. In: Harvey RG (ed) Polycyclic hydrocarbons and carcinogenesis, vol 283, ACS symposium series. American Chemical Society, Washington, DC, pp 85–105
74. Hecht SS, Radok L, Amin S, Huie K, Melikian AA, Hoffmann D, Pataki HRG (1985) Tumorigenicity of 5-methylchrysene dihydrodiols and dihydrodiol epoxides in newborn mice and on mouse skin. Cancer Res 45:1449–1452
75. Flesher JW, Sydnor KL (1973) Possible role of 6-hydroxymethylbenzo[*a*]pyrene as a proximate carcinogen of benzo[*a*]pyrene and 6-methylbenzo[*a*]pyrene. Int J Cancer 11: 433–437
76. Flesher JW, Horn J, Lehner AF (1997) 6-Sulfooxymethylbenzo[*a*]pyrene is an ultimate electrophilic and carcinogenic form of the intermediary metabolite 6-hydroxymethylbenzo-[*a*]pyrene. Biochem Biophys Res Commun 234:554–558
77. Surh YJ, Miller JA (1994) Roles of electrophilic sulfuric acid metabolites in mutagenesis and carcinogenesis by some polynuclear aromatic hydrocarbons. Chem Biol Interact 92:351–362
78. Glatt H (2000) Sulfotransferases in the bioactivation of xenobiotics. Chem Biol Interact 129:141–170
79. Glatt H, Boeing H, Engelke CE, Ma L, Kuhlow A, Pabel U, Pomplun D, Teubner W, Meinl W (2001) Human cytosolic sulphotransferases: genetics, characteristics, toxicological aspects. Mutat Res 482:27–40
80. Surh YJ, Blomquist JC, Miller JA (1991) Activation of 1-hydroxymethylpyrene to an electrophilic and mutagenic metabolite by rat hepatic sulfotransferase activity. Adv Exp Med Biol 283:383–391
81. Miller JA, Surh YJ, Liem A, Miller EC (1991) Electrophilic sulfuric acid ester metabolites of hydroxy-methyl aromatic hydrocarbons as precursors of hepatic benzylic DNA adducts *in vivo*. Adv Exp Med Biol 283:555–567
82. Harris CC (1996) *p53* Tumor suppressor gene: at the crossroad of molecular carcinogenesis, molecular epidemiology and cancer risk assessment. Environ Health Perspect 104:435–439

83. Hoeijmakers JHJ (2001) Genome maintenance mechanisms for preventing cancer. Nature 411:366–374
84. Olivier M, Hussain SP, Caron de Fromentel C, Harris CC (2004) *TP53* mutation spectra and load: a tool for generating hypothesis on the etiology of cancer. IARC Sci Publ 157:247–270
85. Luch A (2005) Nature and nurture—lessons from chemical carcinogenesis. Nat Rev Cancer 5:113–125
86. Luch A (2006) The mode of action of organic carcinogens on cellular structures. EXS 96:65–95
87. Luch A (2002) Cell cycle control and cell division: implications for chemically induced carcinogenesis. ChemBioChem 3:506–516
88. Feng Z, Hu W, Chasin LA, Tang MS (2003) Effects of genomic context and chromatin structure of transcription-coupled and global genomic repair in mammalian cells. Nucleic Acids Res 31:5897–5906
89. Roos WP, Kaina B (2006) DNA damage-induced cell death by apoptosis. Trends Mol Med 12:440–450
90. Marie C, Maitre A, Douki T, Gateau M, Tarantini A, Guiraud P, Favier A, Ravanat JL (2008) Influence of the metabolic properties of human cells on the kinetic of formation of the major benzo[*a*]pyrene DNA adducts. J Appl Toxicol 28:579–590
91. Boysen G, Hecht SS (2003) Analysis of DNA and protein adducts of benzo[*a*]pyrene in human tissues using structure-specific methods. Mutat Res 543:17–30
92. Gyorffy E, Anna L, Gyori Z, Segesdi J, Minárovits J, Soltész I, Kostic S, Csekeo A, Poirier MC, Schoket B (2004) DNA adducts in tumour, normal peripheral lung and bronchus, and peripheral blood lymphocytes from smoking and non-smoking lung cancer patients: correlations between tissues and detection by ^{32}P-postlabelling and immunoassay. Carcinogenesis 25:1201–1209
93. Brinkmann J, Hutzler C, Trappe S, Stolpmann K, Bock U, Liebsch M, Henkler F, Luch A (2010) Comparative studies on the metabolism of benzo[*a*]pyrene in keratinocytes, *in vitro* skin models and human skin *ex vivo*—metabolite patterns as indicator for the metabolic capacity of skin equivalents? Skin-*In-Vitro* 2010, DECHEMA Conference, Frankfurt/Main, Germany, 8–9 Jun 2010, P11
94. Friedberg EC (2001) How nucleotide excision repair protects against cancer. Nat Rev Cancer 1:22–33
95. Friedberg EC (2003) DNA damage and repair. Nature 421:436–440
96. Camenisch U, Naegeli H (2009) Role of DNA repair in the protection against genotoxic stress. In: Luch A (ed) Molecular, clinical and environmental toxicology, vol 1. Birkhäuser, Basel, pp 111–150
97. Naegeli H, Geacintov NE (2005) Mechanisms of repair of polycyclic aromatic hydrocarbon-induced DNA damage. In: Luch A (ed) The carcinogenic effects of polycyclic aromatic hydrocarbons. Imperial College Press, London, pp 211–258
98. Geacintov NE, Broyde S, Buterin T, Naegeli H, Wu M, Yan S, Patel DJ (2002) Thermodynamic and structural factors in the removal of bulky DNA adducts by the nucleotide excision repair machinery. Biopolymers 65:202–210
99. Shuck SC, Short EA, Turchi JJ (2008) Eukaryotic nucleotide excision repair, from understanding mechanisms to influencing biology. Cell Res 18:64–72
100. Hess MT, Gunz D, Luneva N, Geacintov NE, Naegeli H (1997) Base pair conformation-dependent excision of benzo[*a*]pyrene diol epoxide-guanine adducts by human nucleotide excision repair enzymes. Mol Cell Biol 17:7069–7076
101. Buterin T, Hess MT, Luneva N, Geacintov NE, Amin S, Kroth H, Seidel A, Naegeli H (2000) Unrepaired fjord region polycyclic aromatic hydrocarbon-DNA adducts in *ras* codon 61 mutational hot spots. Cancer Res 60:1849–1856
102. Dreij K, Seidel A, Jernström B (2005) Differential removal of DNA adducts derived from anti-diol epoxides of dibenzo[*a,l*]pyrene and benzo[*a*]pyrene in human cells. Chem Res Toxicol 18:655–664

103. Cai Y, Kropachev K, Xu R, Tang Y, Kolbanovskii M, Kolbanovskii A, Amin S, Patel DJ, Broyde S, Geacintov NE (2010) Distant neighbor base sequence context effects in human nucleotide excision repair of a benzo[*a*]pyrene-derived DNA lesion. J Mol Biol 399:397–409
104. Cai Y, Patel DJ, Broyde S, Geacintov NE (2010) Base sequence context effects on nucleotide excision repair. J Nucleic Acids 2010:174252
105. Lagerwerf S, Vrouwe MG, Overmeer RM, Fousteri MI, Mullenders LHF (2011) DNA damage response and transcription. DNA Repair 10:743–750
106. Fousteri M, Mullenders LHF (2008) Transcription-coupled nucleotide excision repair in mammalian cells: molecular mechanisms and biological effects. Cell Res 18:73–84
107. Wittschieben BØ, Wood RD (2003) DDB complexities. DNA Repair 2:1065–1069
108. Wittschieben BØ, Iwai S, Wood RD (2005) DDB1-DDB2 (xeroderma pigmentosum group E) protein complex recognizes a cyclobutane pyrimidine dimer, mismatches, apurinic/apyrimidinic sites, and compound lesions in DNA. J Biol Chem 280:39982–39989
109. Mitra S, Izumi T, Boldogh I, Bhakat KK, Hill JW, Hazra TK (2002) Choreography of oxidative damage repair in mammalian genomes. Free Radic Biol Med 33:15–28
110. Hedge ML, Hazra TK, Mitra S (2008) Early steps in the DNA base excision/single-strand interruption repair pathway in mammalian cells. Cell Res 18:27–47
111. Mitra S, Boldogh I, Izumi T, Hazra TK (2001) Complexities of the DNA base excision repair pathway for repair of oxidative DNA damage. Environ Mol Mutagen 38:180–190
112. Gros L, Saparbaev MK, Laval J (2002) Enzymology of the repair of free radicals-induced DNA damage. Oncogene 21:8905–8925
113. Wu J, Gu L, Wang H, Geacintov NE, Li GM (1999) Mismatch repair processing of carcinogen-DNA adducts triggers apoptosis. Mol Cell Biol 19:8292–8301
114. Vaisman A, Masutani C, Hanaoka F, Chaney SG (2000) Efficient translesion replication past oxaliplatin and cisplatin GpG adducts by human DNA polymerase η. Biochemistry 39: 4575–4580
115. Colussi C, Parlanti E, Degan P, Aquilina G, Barnes D, Macpherson P, Karran P, Crescenzi M, Dogliotti E, Bignami M (2002) The mammalian mismatch repair pathway removes DNA 8-oxodGMP incorporated from the oxidized dNTP pool. Curr Biol 12:912–918
116. Christmann M, Tomicic MT, Roos WP, Kaina B (2003) Mechansims of human DNA repair: an update. Toxicology 193:3–34
117. Young LC, Keuling AM, Lai R, Nation PN, Tron VA, Andrew SE (2007) The associated contributions of p53 and the DNA mismatch repair protein Msh6 to spontaneous tumorigenesis. Carcinogenesis 28:2131–2138
118. Stout GJ, van Oosten M, Acherrat FZ, de Wit J, Vermeij WP, Mullenders LHF, de Gruijl FR, Backendorf C (2005) Selective DNA damage responses in murine $Xpa^{-/-}$, $Xpc^{-/-}$ and $Csb^{-/-}$ keratinocyte cultures. DNA Repair 4:1337–1344
119. Stuart D, Khan QA, Brown R, Dipple A (2001) Hydrocarbon carcinogens induce p53 activity in normal mouse tissue. Cancer Lett 173:111–114
120. Pääjärvi G, Jernström B, Seidel A, Stenius U (2008) Anti-diol epoxide of benzo*[a]*pyrene induces transient Mdm2 and p53 Ser15 phosphorylation, while anti-diol epoxide of dibenzo-[*a*,*l*]pyrene induces a nontransient p53 Ser15 phosphorylation. Mol Carcinog 47:301–309
121. Bhana S, Lloyd DR (2008) The role of p53 in DNA damage-mediated cytotoxicity overrides its ability to regulate nucleotide excision repair in human fibroblasts. Mutagenesis 23:43–50
122. Hahn ME, Allan LA, Sherr DH (2009) Regulation of constitutive and inducible AHR signaling: complex interactions involving the AHR repressor. Biochem Pharmacol 77: 485–497
123. Duchaud E, Ridet A, Stoppa-Lyonnet D, Janin N, Moustacchi E, Rosselli F (1996) Deregulated apoptosis in ataxia telangiectasia: association with clinical stigmata and radiosensitivity. Cancer Res 56:1400–1404
124. Norbury CJ, Zhivotovsky B (2004) DNA damage-induced apoptosis. Oncogene 23: 2797–2808
125. Yhivotovsky B, Kroemer G (2004) Apoptosis and genomic instability. Nat Rev Mol Cell Biol 5:752–762

126. Al-Ejeh F, Kumar R, Wiegmans A, Lakhani SR, Brown MP, Khanna KK (2010) Harnessing the complexity of DNA-damage response pathways to improve cancer treatment outcomes. Oncogene 29:6085–6098
127. Hartley KO, Gell D, Smith GC, Zhang H, Divecha N, Connelly MA, Admon A, Lees-Miller SP, Anderson CW, Jackson SP (1995) DNA-dependent protein kinase catalytic subunit: a relative of phosphatidylinositol 3-kinase and the ataxia telangiectasia gene product. Cell 82:849–856
128. Smith J, Tho LM, Xu N, Gillespie DA (2010) The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer. Adv Cancer Res 108:73–112
129. Vaziri C, Faller DV (1997) A benzo[*a*]pyrene-induced cell cycle checkpoint resulting in p53-independent G1 arrest in 3T3 fibroblasts. J Biol Chem 272:2762–2769
130. Guo N, Faller DV, Vaziri C (2002) Carcinogen-induced S-phase arrest is Chk1 mediated and caffeine sensitive. Cell Growth Differ 13:77–86
131. Bi X, Slater DM, Ohmori H, Vaziri C (2005) DNA polymerase κ is specifically required for recovery from the benzo[*a*]pyrene-dihydrodiol epoxide (BPDE)-induced S-phase checkpoint. J Biol Chem 280:22343–22355
132. Caino MC, Oliva JL, Jiang H, Penning TM, Kazanietz MG (2007) Benzo[*a*]pyrene-7, 8-dihydrodiol promotes checkpoint activation and G2/M arrest in human bronchoalveolar carcinoma H358 cells. Mol Pharmacol 71:744–750
133. Ford JM (2005) Regulation of DNA damage recognition and nucleotide excision repair: another role for p53. Mutat Res 577:195–202
134. Adimoolam S, Ford JM (2002) p53 and DNA damage-inducible expression of the xeroderma pigmentosum group C gene. Proc Natl Acad Sci USA 99:12985–12990
135. Adimoolam S, Ford JM (2003) p53 and regulation of DNA damage recognition during nucleotide excision repair. DNA Repair 18:947–954
136. Lloyd DR, Hanawalt PC (2000) p53-dependent global genomic repair of benzo[*a*]pyrene-7,8-diol-9,10-epoxide adducts in human cells. Cancer Res 60:517–521
137. Lloyd DR, Hanawalt PC (2002) p53 controls global nucleotide excision repair of low levels of structurally diverse benzo[*g*]chrysene-DNA adducts in human fibroblasts. Cancer Res 62:5288–5294
138. Hanawalt PC, Ford JM, Lloyd DR (2003) Functional characterization of global genomic DNA repair and its implications for cancer. Mutat Res 544:107–114
139. Luch A, Kudla K, Seidel A, Doehmer J, Greim H, Baird WM (1999) The level of DNA modification by (+)-*syn*-(11*S*,12*R*,13*S*,14*R*)- and (–)-*anti*-(11*R*,12*S*,13*S*,14*R*)-dihydrodiol epoxides of dibenzo[*a,l*]pyrene determined the effect on the proteins p53 and $p21^{WAF1}$ in the human mammary carcinoma cell line MCF-7. Carcinogenesis 20:859–865
140. Nakanishi Y, Pei XH, Takayama K, Bai F, Izumi M, Kimotsuki K, Inoue K, Minami T, Wataya H, Hara N (2000) Polycyclic aromatic hydrocarbon carcinogens increase ubiquitination of p21 protein after the stabilization of p53 and the expression of p21. Am J Respir Cell Mol Biol 22:747–754
141. Högberg J, Silins I, Stenius U (2009) Chemical induced alterations in p53 signaling. In: Luch A (ed) Molecular, clinical and environmental toxicology, vol 1. Birkhäuser, Basel, pp 181–208
142. Auclair Y, Rouget R, El Affar B, Drobetsky EA (2008) ATR kinase is required for global genomic nucleotide excision repair exclusively during S phase in human cells. Proc Natl Acad Sci USA 105:17896–17901
143. Auclair Y, Rouget R, Drobetsky EA (2009) ATR kinase as master regulator of nucleotide excision repair during S phase of the cell cycle. Cell Cycle 8:1865–1871
144. Colton SL, Xu XS, Wang YA, Wang G (2006) The involvement of ataxia-telangiectasia mutated protein activation in nucleotide excision repair-facilitated cell survival with cisplatin treatment. J Biol Chem 281:27117–27125
145. Wu X, Shell SM, Zou Y (2005) Interaction and colocalization of Rad9/Rad1/Hus1 checkpoint complex with replication protein A in human cells. Oncogene 24:4728–4735
146. Wu X, Shell SM, Liu Y, Zou Y (2007) ATR-dependent checkpoint modulates XPA nuclear import in response to UV irradiation. Oncogene 26:757–764

147. Wu X, Shell SM, Yang Z, Zou Y (2006) Phosphorylation of nucleotide excision repair factor xeroderma pigmentosum group A by ataxia telangiectasia mutated and Rad3-related-dependent checkpoint pathway promotes cell survival in response to UV irradiation. Cancer Res 66:2997–3005
148. Ko CB, Kim SJ, Park C, Kim BR, Shin CH, Choi S, Chung SY, Noh JH, Jeun JH, Kim NS, Park R (2004) Benzo[*a*]pyrene-induced apoptotic death of mouse hepatoma Hepa1c1c7 cells via activation of intrinsic caspase cascade and mitochondrial dysfunction. Toxicology 199:35–46
149. Kim SJ, Ko CB, Park C, Kim BR, Sung TH, Koh DH, Kim NS, Oh KJ, Chung SY, Park R (2005) p38 MAP kinase regulates benzo[*a*]pyrene-induced apoptosis through th regulation of p53 activation. Arch Biochem Biophys 444:121–129
150. Chen S, Nguyen N, Tamura K, Karin M, Tukey RH (2003) The role of the Ah receptor and p38 in benzo[*a*]pyrene-7,8-dihydrodiol and benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide-induced apoptosis. J Biol Chem 278:19526–19533
151. Lin T, Mak NK, Yang MS (2008) MAPK regulate p53-dependent cell death induced by benzo[*a*]pyrene: involvement of p53 phosphorylation and acetylation. Toxicology 247: 145–153
152. Bulavin DV, Saito S, Hollander MC, Sakaguchi K, Anderson CW, Appella E, Fornace AJ Jr (1999) Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. EMBO J 18:6845–6854
153. Bulavin DV, Fornace AJ Jr (2004) p38 MAP kinase's emerging role as a tumor suppressor. Adv Cancer Res 92:95–118
154. Barkley LR, Ohmori H, Vaziri C (2007) Integrating S-phase checkpoint signaling with trans-lesion synthesis of bulky DNA adducts. Cell Biochem Biophys 47:392–408
155. Wani MA, Zhu Q, El-Mahdy M, Venkatachalam S, Wani AA (2000) Enhanced sensitivity to *anti*-benzo[*a*]pyrene-diol-epoxide DNA damage correlates with decreased global genomic repair attributable to abrogated p53 function in human cells. Cancer Res 60:2273–2280
156. Paules RS, Cordeiro-Stone M, Mass MJ, Poirier MC, Yuspa SH, Kaufman DG (1988) Benzo[*a*]pyrene diol epoxide I binds to DNA at replication forks. Proc Natl Acad Sci USA 85:2176–2180
157. Sidi S, Sanda T, Kennedy RD, Hagen AT, Jette CA, Hoffmans R, Pascual J, Imamura S, Kishi S, Amatruda JF, Kanki JP, Green DR, D'Andrea AA, Look AT (2008) Chk1 suppresses a caspase-2 apoptotic response to DNA damage that bypasses p53, Bcl-2, and caspase-3. Cell 133:864–877
158. Olson JM, Hallahan AR (2004) p38 MAP kinase: a convergence point in cancer therapy. Trends Mol Med 10:125–129
159. Galan-Moya EM, Hernandez-Losa J, Aceves Luquero CI, de la Cruz-Morcillo MA, Ramírez-Castillejo C, Callejas-Valera JL, Arriaga A, Aranburo AF, Ramón y Cajal S, Gutkind JS, Sánchez-Prieto R (2008) c-Abl activates p38 MAPK independently of its tyrosine kinase activity: implications in cisplatin-based therapy. Int J Cancer 122: 289–297
160. Huc L, Sparfel L, Rissel M, Dimanche-Boitrel MT, Guillouzo A, Fardel O, Lagadic-Gossmann D (2004) Identification of Na^+/H^+ exchange as a new target for toxic polycyclic aromatic hydrocarbons. FASEB J 18:344–346
161. Huc L, Rissel M, Solhaug A, Tekpli X, Gorria M, Torriglia A, Holme JA, Dimanche-Boitrel MT, Lagadic-Gossmann D (2006) Multiple apoptotic pathways induced by p53-dependent acidification in benzo[*a*]pyrene-exposed hepatic F258 cells. J Cell Physiol 208:527–537
162. Huc L, Tekpli X, Holme JA, Rissel M, Solhaug A, Gardyn C, Le Moigne G, Gorria M, Dimanche-Boitrel MT, Lagadic-Gossmann D (2007) c-Jun NH_2-terminal kinase-related Na^+/H^+ exchanger isoform 1 activation controls hexokinase II expression in benzo[*a*]pyrene-induced apoptosis. Cancer Res 67:1696–1705
163. Burdick AD, Davis JW 2nd, Liu KJ, Hudson LG, Shi H, Monske ML, Burchiel SW (2003) Benzo[*a*]pyrene quinones increase cell proliferation, generate reactive oxygen species, and

transactivate the epidermal growth factor receptor in breast epithelial cells. Cancer Res 63:7825–7833
164. Burdick AD, Ivnitski-Steele ID, Lauer FT, Burchiel SW (2006) PYK2 mediates anti-apoptotic AKT signaling in response to benzo[*a*]pyrene diol epoxide in mammary epithelial cells. Carcinogenesis 27:2331–2340
165. Tannheimer SL, Ethier SP, Caldwell KK, Burchiel SW (1998) Benzo[*a*]pyrene- and TCDD-induced alterations in tyrosine phosphorylation and insulin-like growth factor signaling pathways in the MCF-10A human mammary epithelial cell line. Carcinogenesis 19: 1291–1297
166. Rodriguez-Fragoso L, Melendez K, Hudson LG, Lauer FT, Burchiel SW (2009) EGF-receptor phosphorylation and downstream signaling are activated by benzo[*a*]pyrene 3,6-quinone and benzo[*a*]pyrene 1,6-quinone in human mammary epithelial cells. Toxicol Appl Pharmacol 235:321–328
167. Pourazar J, Blomberg KFJ, Davies DE, Wilson SJ, Holgate ST, Sandström T (2008) Diesel exhaust increases EGFR and phosphorylated C-terminal Tyr 1173 in the bronchial epithelium. Part Fibre Toxicol 5:8–17
168. Rhee SG (2006) Cell signaling. H_2O_2, a necessary evil for cell signaling. Science 312: 1882–1883
169. Lecureur V, Ferrec EL, N'diaye M, Vee ML, Gardyn C, Gilot D, Fardel O (2005) ERK-dependent induction of TNFα expression by the environmental contaminant benzo[*a*]pyrene in primary human macrophages. FEBS Lett 579:1904–1910
170. Umannová L, Machala M, Topinka J, Nováková Z, Milcová A, Kozubík A, Vondrácek J (2008) Tumor necrosis factor-α potentiates genotoxic effects of benzo[*a*]pyrene in rat liver epithelial cells through upregulation of cytochrome P450 1B1 expression. Mutat Res 640: 162–169
171. Umannová L, Zatloukalová J, Machala M, Krčmář P, Májková Z, Hennig B, Kozubík A, Vondrácek J (2007) Tumor necrosis factor-α modulates effects of aryl hydrocarbon receptor ligands on cell proliferation and expression of cytochrome P450 enzymes in rat liver "stem-like" cells. Toxicol Sci 99:79–89
172. Umannová L, Machala M, Topinka J, Schmuczerová J, Krčmář P, Neča J, Šujanová K, Kozubík A, Vondrácek J (2011) Benzo[*a*]pyrene and tumor necrosis factor-α coordinately increase genotoxic damage and the production of proinflammatory mediators in alveolar epithelial type II cells. Toxicol Lett 206:121–129
173. Barouki R, Coumoul X, Fernandez-Salguero PM (2007) The aryl hydrocarbon receptor, more than a xenobiotic-interacting protein. FEBS Lett 58:3608–3615
174. Curfs DM, Knaapen AM, Pachen DM, Gijbels MJ, Lutgens E, Smook ML, Kockx MM, Daemen MJ, van Schooten FJ (2005) Polycyclic aromatic hydrocarbons induce an inflammatory atherosclerotic plaque phenotype irrespective of their DNA binding properties. FASEB J 19:1290–1292
175. Kepley CL, Lauer FT, Oliver JM, Burchiel SW (2003) Environmental polycyclic aromatic hydrocarbons, benzo[*a*]pyrene (BaP) and BaP-quinones, enhance IgE-mediated histamine release and IL-4 production in human basophils. Clin Immunol 107:10–19
176. Veldhoen M, Hirota K, Westendorf AM, Buer J, Dumoutier L, Renauld JC, Stockinger B (2008) The aryl hydrocarbon receptor links T_H17-cell-mediated autoimmunity to environmental toxins. Nature 453:106–109
177. Stolpmann K, Brinkmann J, Salzmann S, Genkinger D, Fritsche E, Hutzler C, Wajant H, Luch A, Henkler F (2011) Activation of the aryl hydrocarbon receptor sensitizes human keratinocytes for CD95L- and TRAIL-induced apoptosis. Submitted

Heavy Metal Toxicity and the Environment

Paul B. Tchounwou, Clement G. Yedjou, Anita K. Patlolla, and Dwayne J. Sutton

Abstract Heavy metals are naturally occurring elements that have a high atomic weight and a density at least five times greater than that of water. Their multiple industrial, domestic, agricultural, medical, and technological applications have led to their wide distribution in the environment, raising concerns over their potential effects on human health and the environment. Their toxicity depends on several factors including the dose, route of exposure, and chemical species, as well as the age, gender, genetics, and nutritional status of exposed individuals. Because of their high degree of toxicity, arsenic, cadmium, chromium, lead, and mercury rank among the priority metals that are of public health significance. These metallic elements are considered systemic toxicants that are known to induce multiple organ damage, even at lower levels of exposure. They are also classified as human carcinogens (known or probable) according to the US Environmental Protection Agency and the International Agency for Research on Cancer. This review provides an analysis of their environmental occurrence, production and use, potential for human exposure, and molecular mechanisms of toxicity, genotoxicity, and carcinogenicity.

Keywords Carcinogenicity · Genotoxicity · Heavy metals · Human exposure · Production and use · Toxicity

P. B. Tchounwou (✉), C. G. Yedjou, A. K. Patlolla and D. J. Sutton
NIH-RCMI Center for Environmental Health, College of Science, Engineering and Technology, Jackson State University, 1400 Lynch Street, Box 18750, Jackson, MS 39217, USA
e-mail: paul.b.tchounwou@jsums.edu

A. Luch (ed.), *Molecular, Clinical and Environmental Toxicology*,
Experientia Supplementum 101, DOI 10.1007/978-3-7643-8340-4_6,

Introduction

Heavy metals are defined as metallic elements that have a relatively high density compared to water [1]. With the assumption that heaviness and toxicity are interrelated, heavy metals also include metalloids, such as arsenic, that are able to induce toxicity at low level of exposure [2]. In recent years, there has been an increasing ecological and global public health concern associated with environmental contamination by these metals. Also, human exposure has risen dramatically as a result of an exponential increase of their use in several industrial, agricultural, domestic, and technological applications [3]. Reported sources of heavy metals in the environment include geogenic, industrial, agricultural, pharmaceutical, domestic effluents, and atmospheric sources [4]. Environmental pollution is very prominent in point source areas such as mining, foundries and smelters, and other metal-based industrial operations [1, 3, 4].

Although heavy metals are naturally occurring elements that are found throughout the earth's crust, most environmental contamination and human exposure result from anthropogenic activities such as mining and smelting operations, industrial production and use, and domestic and agricultural use of metals and metal-containing compounds [4–7]. Environmental contamination can also occur through metal corrosion, atmospheric deposition, soil erosion of metal ions and leaching of heavy metals, sediment resuspension, and metal evaporation from water resources to soil and groundwater [8]. Natural phenomena such as weathering and volcanic eruptions have also been reported to significantly contribute to heavy metal pollution [1, 3, 4, 7, 8]. Industrial sources include metal processing in refineries, coal burning in power plants, petroleum combustion, nuclear power stations and high tension lines, plastics, textiles, microelectronics, wood preservation, and paper-processing plants [9–11].

It has been reported that metals such as cobalt (Co), copper (Cu), chromium (Cr), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se), and zinc (Zn) are essential nutrients that are required for various biochemical and physiological functions [12]. Inadequate supply of these micronutrients results in a variety of deficiency diseases or syndromes [12].

Heavy metals are also considered as trace elements because of their presence in trace concentrations (ppb range to less than 10 ppm) in various environmental matrices [13]. Their bioavailability is influenced by physical factors such as temperature, phase association, adsorption, and sequestration. It is also affected by chemical factors that influence speciation at thermodynamic equilibrium, complexation kinetics, lipid solubility, and octanol/water partition coefficients [14]. Biological factors, such as species characteristics, trophic interactions, and biochemical/physiological adaptation, also play an important role [15].

The essential heavy metals exert biochemical and physiological functions in plants and animals. They are important constituents of several key enzymes and play important roles in various oxidation–reduction reactions [12]. Copper, for example, serves as an essential cofactor for several oxidative stress-related

enzymes including catalase, superoxide dismutase, peroxidase, cytochrome c oxidases, ferroxidases, monoamine oxidase, and dopamine β-monooxygenase [16–18]. Hence, it is an essential nutrient that is incorporated into a number of metalloenzymes involved in hemoglobin formation, carbohydrate metabolism, catecholamine biosynthesis, and cross-linking of collagen, elastin, and hair keratin. The ability of copper to cycle between an oxidized state, Cu(II), and reduced state, Cu(I), is used by cuproenzymes involved in redox reactions [16–18]. However, it is this property of copper that also makes it potentially toxic because the transitions between Cu(II) and Cu(I) can result in the generation of superoxide and hydroxyl radicals [16–19]. Also, excessive exposure to copper has been linked to cellular damage leading to Wilson disease in humans [18, 19]. Similar to copper, several other essential elements are required for biologic functioning; however, an excess amount of such metals produces cellular and tissue damage leading to a variety of adverse effects and human diseases. For some including chromium and copper, there is a very narrow range of concentrations between beneficial and toxic effects [19, 20]. Other metals such as aluminum (Al), antinomy (Sb), arsenic (As), barium (Ba), beryllium (Be), bismuth (Bi), cadmium (Cd), gallium (Ga), germanium (Ge), gold (Au), indium (In), lead (Pb), lithium (Li), mercury (Hg), nickel (Ni), platinum (Pt), silver (Ag), strontium (Sr), tellurium (Te), thallium (Tl), tin (Sn), titanium (Ti), vanadium (V), and uranium (U) have no established biological functions and are considered as nonessential metals [20].

In biological systems, heavy metals have been reported to affect cellular organelles and components such as cell membrane, mitochondrial, lysosome, endoplasmic reticulum, nuclei, and some enzymes involved in metabolism, detoxification, and damage repair [21]. Metal ions have been found to interact with cell components such as DNA and nuclear proteins, causing DNA damage and conformational changes that may lead to cell-cycle modulation, carcinogenesis, or apoptosis [20–22]. Several studies from our laboratory have demonstrated that reactive oxygen species (ROS) production and oxidative stress play a key role in the toxicity and carcinogenicity of metals such as arsenic [23–25], cadmium [26], chromium [27, 28], lead [29, 30], and mercury [31, 32]. Because of their high degree of toxicity, these five elements rank among the priority metals that are of great public health significance. They are all systemic toxicants that are known to induce multiple organ damage, even at lower levels of exposure. According to the US Environmental Protection Agency (US EPA) and the International Agency for Research on Cancer (IARC), these metals are also classified as either "known" or "probable" human carcinogens based on epidemiological and experimental studies showing an association between exposure and cancer incidence in humans and animals.

Heavy metal-induced toxicity and carcinogenicity involve many mechanistic aspects, some of which are not clearly elucidated or understood. However, each metal is known to have unique features and physicochemical properties that confer to its specific toxicological mechanisms of action. This review provides an analysis of the environmental occurrence, production and use, potential for human exposure, and molecular mechanisms of toxicity, genotoxicity, and carcinogenicity of arsenic, cadmium, chromium, lead, and mercury.

Arsenic

Environmental Occurrence, Industrial Production and Use

Arsenic is a ubiquitous element that is detected at low concentrations in virtually all environmental matrices [33]. The major inorganic forms of arsenic include the trivalent arsenite and the pentavalent arsenate. The organic forms are the methylated metabolites—monomethylarsonic acid (MMA), dimethylarsonic acid (DMA), and trimethylarsine oxide. Environmental pollution by arsenic occurs as a result of natural phenomena such as volcanic eruptions and soil erosion and anthropogenic activities [33]. Several arsenic-containing compounds are produced industrially and have been used to manufacture products with agricultural applications such as insecticides, herbicides, fungicides, algicides, sheep dips, wood preservatives, and dyestuffs. They have also been used in veterinary medicine for the eradication of tapeworms in sheep and cattle [34]. Arsenic compounds have also been used in the medical field for at least a century in the treatment of syphilis, yaws, amoebic dysentery, and trypanosomiasis [34, 35]. Arsenic-based drugs are still used in treating certain tropical diseases such as African sleeping sickness and amoebic dysentery and in veterinary medicine to treat parasitic diseases, including filariasis in dogs and blackhead in turkeys and chickens [35]. Recently, arsenic trioxide has been approved by the Food and Drug Administration as an anticancer agent in the treatment of acute promyelocytic leukemia [36]. Its therapeutic action has been attributed to the induction of programmed cell death (apoptosis) in leukemia cells [24].

Potential for Human Exposure

It is estimated that several million people are exposed to arsenic chronically throughout the world, especially in countries like Bangladesh, India, Chile, Uruguay, Mexico, and Taiwan, where the groundwater is contaminated with high concentrations of arsenic. Exposure to arsenic occurs via the oral route (ingestion), inhalation, dermal contact, and the parenteral route to some extent [33, 34, 37]. Arsenic concentrations in air range from 1 to 3 ng/m^3 in remote locations (away from human releases) and from 20 to 100 ng/m^3 in cities. Its water concentration is usually less than 10 μg/L, although higher levels can occur near natural mineral deposits or mining sites. Its concentration in various foods ranges from 20 to 140 ng/kg [38]. Natural levels of arsenic in soil usually range from 1 to 40 mg/kg, but pesticide application or waste disposal can produce much higher values [25].

Diet, for most individuals, is the largest source of exposure, with an average intake of about 50 μg per day. Intake from air, water, and soil is usually much smaller, but exposure from these media may become significant in areas of

arsenic contamination. Workers who produce or use arsenic compounds in such occupations as vineyards, ceramics, glassmaking, smelting, refining of metallic ores, pesticide manufacturing and application, wood preservation, and semiconductor manufacturing can be exposed to substantially higher levels of arsenic [39]. Arsenic has also been identified at 781 sites of the 1,300 hazardous waste sites that have been proposed by the US EPA for inclusion on the national priority list [33, 39]. Human exposure at these sites may occur by a variety of pathways, including inhalation of dusts in air, ingestion of contaminated water or soil, or through the food chain [40].

Contamination with high levels of arsenic is of concern because arsenic can cause a number of human health effects. Several epidemiological studies have reported a strong association between arsenic exposure and increased risks of both carcinogenic and systemic health effects [41]. Interest in the toxicity of arsenic has been heightened by recent reports of large populations in West Bengal, Bangladesh, Thailand, Inner Mongolia, Taiwan, China, Mexico, Argentina, Chile, Finland, and Hungary that have been exposed to high concentrations of arsenic in their drinking water and are displaying various clinicopathological conditions including cardiovascular and peripheral vascular disease, developmental anomalies, neurologic and neurobehavioral disorders, diabetes, hearing loss, portal fibrosis, hematologic disorders (anemia, leukopenia, and eosinophilia), and carcinoma [25, 33, 35, 39]. Arsenic exposure affects virtually all organ systems including the cardiovascular, dermatologic, nervous, hepatobiliary, renal, gastrointestinal, and respiratory systems [41]. Research has also pointed to significantly higher standardized mortality rates for cancers of the bladder, kidney, skin, and liver in many areas of arsenic pollution. The severity of adverse health effects is related to the chemical form of arsenic and is also time and dose dependent [42, 43]. Although the evidence of carcinogenicity of arsenic in humans seems strong, the mechanism by which it produces tumors in humans is not completely understood [44].

Molecular Mechanisms of Toxicity and Carcinogenicity

Analyzing the toxic effects of arsenic is complicated because the toxicity is highly influenced by its oxidation state and solubility, as well as many other intrinsic and extrinsic factors [45]. Several studies have indicated that the toxicity of arsenic depends on the exposure dose, frequency and duration, the biological species, age, and gender, as well as on individual susceptibilities and genetic and nutritional factors [46]. Most cases of human toxicity from arsenic have been associated with exposure to inorganic arsenic. Inorganic trivalent arsenite [As(III)] is 2–10 times more toxic than pentavalent arsenate [As(V)] [5]. By binding to thiol or sulfhydryl groups on proteins, As(III) can inactivate over 200 enzymes. This is the likely mechanism responsible for arsenic's widespread effects on different organ systems. As(V) can replace phosphate, which is involved in many biochemical pathways [5, 47].

One of the mechanisms by which arsenic exerts its toxic effect is through impairment of cellular respiration by the inhibition of various mitochondrial enzymes and the uncoupling of oxidative phosphorylation. Most toxicity of arsenic results from its ability to interact with sulfhydryl groups of proteins and enzymes and to substitute phosphorous in a variety of biochemical reactions [48]. Arsenic *in vitro* reacts with protein sulfhydryl groups to inactivate enzymes, such as dihydrolipoyl dehydrogenase and thiolase, thereby producing inhibited oxidation of pyruvate and beta-oxidation of fatty acids [49]. The major metabolic pathway for inorganic arsenic in humans is methylation. Arsenic trioxide is methylated to two major metabolites via a nonenzymatic process to MMA, which is further methylated enzymatically to DMA before excretion in the urine [40, 47]. It was previously thought that this methylation process is a pathway of arsenic detoxification; however, recent studies have pointed out that some methylated metabolites may be more toxic than arsenite if they contain trivalent forms of arsenic [41].

Tests for genotoxicity have indicated that arsenic compounds inhibit DNA repair and induce chromosomal aberrations, sister chromatid exchanges, and micronuclei formation in both human and rodent cells in culture [50–52] and in cells of exposed humans [53]. Reversion assays with *Salmonella typhimurium* fail to detect mutations that are induced by arsenic compounds. Although arsenic compounds are generally perceived as weak mutagens in bacterial and animal cells, they exhibit clastogenic properties in many cell types *in vivo* and *in vitro* [54]. In the absence of animal models, *in vitro* cell transformation studies become a useful means of obtaining information on the carcinogenic mechanisms of arsenic toxicity. Arsenic and arsenical compounds are cytotoxic and induce morphological transformations of Syrian hamster embryo (SHE) cells as well as mouse C3H10T1/2 cells and BALB/3T3 cells [55, 56].

Based on the comet assay, it has been reported that arsenic trioxide induces DNA damage in human lymphocytes [57] and also in mouse leukocytes [58]. Arsenic compounds have also been shown to induce gene amplification, arrest cells in mitosis, inhibit DNA repair, and induce expression of the *c-fos* gene and the oxidative stress protein heme oxygenase in mammalian cells [52, 58]. They have been implicated as promoters and comutagens for a variety of toxic agents [59]. Recent studies in our laboratory have demonstrated that arsenic trioxide is cytotoxic and able to transcriptionally induce a significant number of stress genes and related proteins in human liver carcinoma cells [60].

Epidemiological investigations have indicated that long-term arsenic exposure results in promotion of carcinogenesis. Several hypotheses have been proposed to describe the mechanism of arsenic-induced carcinogenesis. Zhao *et al.* [61] reported that arsenic may act as a carcinogen by inducing DNA hypomethylation, which in turn facilitates aberrant gene expression. Additionally, it was found that arsenic is a potent stimulator of extracellular signal-regulated protein kinase Erk1 and AP-1 transactivational activity and an efficient inducer of *c-fos* and *c-jun* gene expression [62]. Induction of *c-jun* and *c-fos* by arsenic is associated with activation

of JNK [63]. However, the role of JNK activation by arsenite in cell transformation or tumor promotion is unclear.

In another study, Trouba *et al*. [64] concluded that long-term exposure to high levels of arsenic might make cells more susceptible to mitogenic stimulation and that alterations in mitogenic signaling proteins might contribute to the carcinogenic action of arsenic. Collectively, several recent studies have demonstrated that arsenic can interfere with cell signaling pathways (e.g., the p53 signaling pathway) that are frequently implicated in the promotion and progression of a variety of tumor types in experimental animal models and of some human tumors [65, 66, 67]. However, the specific alterations in signal transduction pathways or the actual targets that contribute to the development of arsenic-induced tumors in humans following chronic consumption of arsenic remain uncertain.

Recent clinical trials have found that arsenic trioxide has therapeutic value in the treatment of acute promyelocytic leukemia, and there is interest in exploring its effectiveness in the treatment of a variety of other cancers [68, 69]. In acute promyelocytic leukemia, the specific molecular event critical to the formation of malignant cells is known. A study by Puccetti *et al*. [70] found that forced over-expression of BCR-ABL susceptibility in human lymphoblasts cells resulted in greatly enhanced sensitivity to arsenic-induced apoptosis. They also concluded that arsenic trioxide is a tumor-specific agent capable of inducing apoptosis selectively in acute promyelocytic leukemia cells. Several recent studies have shown that arsenic can induce apoptosis through alterations in other cell signaling pathways [71, 72]. In addition to acute promyelocytic leukemia, arsenic is thought to have therapeutic potential for myeloma [73]. In summary, numerous cancer chemotherapy studies in cell cultures and in patients with acute promyelocytic leukemia demonstrate that arsenic trioxide administration can lead to cell-cycle arrest and apoptosis in malignant cells.

Previous studies have also examined p53 gene expression and mutation in tumors obtained from subjects with a history of arsenic ingestion. p53 participates in many cellular functions, cell-cycle control, DNA repair, differentiation, genomic plasticity, and programmed cell death. Additional support for the hypothesis that arsenic can modulate gene expression has been provided by several different studies [74, 75]. Collectively, these studies provide further evidence that various forms of arsenic can alter gene expression and that such changes could contribute substantially to the toxic and carcinogenic actions of arsenic treatment in human populations [76].

Several *in vitro* studies in our laboratory have demonstrated that arsenic modulates DNA synthesis, gene and protein expression, genotoxicity, mitosis, and/or apoptotic mechanisms in various cell lines including keratinocytes, melanocytes, dendritic cells, dermal fibroblasts, microvascular endothelial cells, monocytes and T cells [77], colon cancer cells [78], lung cancer cells [79], human leukemia cells [80], Jurkat-T lymphocytes [81], and human liver carcinoma cells [82]. We have also shown that oxidative stress plays a key role in arsenic-induced cytotoxicity, a process that is modulated by pro- and/or antioxidants such as ascorbic acid and *N*-acetyl cysteine [43, 83, 84]. We have further demonstrated that the toxicity of

arsenic depends on its chemical form, the inorganic form being more toxic than the organic one [42].

Various hypotheses have been proposed to explain the carcinogenicity of inorganic arsenic. Nevertheless, the molecular mechanisms by which this arsenic induces cancer are still poorly understood. Results of previous studies have indicated that inorganic arsenic does not act through classic genotoxic and mutagenic mechanisms, but rather may be a tumor promoter that modifies signal transduction pathways involved in cell growth and proliferation [67]. Although much progress has been recently made in the area of arsenic's possible mode(s) of carcinogenic action, a scientific consensus has not yet reached. A recent review discusses nine different possible modes of action of arsenic carcinogenesis: induced chromosomal abnormalities, oxidative stress, altered DNA repair, altered DNA methylation patterns, altered growth factors, enhanced cell proliferation, promotion/progression, suppression of p53, and gene amplification [85]. Presently, three modes (chromosomal abnormality, oxidative stress, and altered growth factors) of arsenic carcinogenesis have shown a degree of positive evidence, both in experimental systems (animal and human cells) and in human tissues. The remaining possible modes of carcinogenic action (progression of carcinogenesis, altered DNA repair, p53 suppression, altered DNA methylation patterns, and gene amplification) do not have as much evidence, particularly from *in vivo* studies with laboratory animals, *in vitro* studies with cultured human cells, or human data from case or population studies. Thus, the mode-of-action studies suggest that arsenic might be acting as a cocarcinogen, a promoter, or a progressor of carcinogenesis.

Cadmium

Environmental Occurrence, Industrial Production and Use

Cadmium is a heavy metal of considerable environmental and occupational concern. It is widely distributed in the earth's crust at an average concentration of about 0.1 mg/kg. The highest level of cadmium compounds in the environment is accumulated in sedimentary rocks, and marine phosphates contain about 15 mg cadmium/kg [86].

Cadmium is frequently used in various industrial activities. The major industrial applications of cadmium include the production of alloys, pigments, and batteries [87]. Although the use of cadmium in batteries has shown considerable growth in recent years, its commercial use has declined in developed countries in response to environmental concerns. In the United States, for example, the daily cadmium intake is about 0.4 μg/kg/day, less than half of the US EPA's oral reference dose [88]. This decline has been linked to the introduction of stringent effluent limits from plating works and, more recently, to the introduction of general restrictions on cadmium consumption in certain countries.

Potential for Human Exposure

The main routes of exposure to cadmium are via inhalation or cigarette smoke and ingestion of food. Skin absorption is rare. Human exposure to cadmium is possible through a number of several sources including employment in primary metal industries, eating contaminated food, smoking cigarettes, and working in cadmium-contaminated workplaces, with smoking being a major contributor [89, 90]. Other sources of cadmium include emissions from industrial activities, including mining, smelting, and manufacturing of batteries, pigments, stabilizers, and alloys [91]. Cadmium is also present in trace amounts in certain foods such as leafy vegetables, potatoes, grains and seeds, liver and kidney, and crustaceans and mollusks [92]. In addition, foodstuffs that are rich in cadmium can greatly increase the cadmium concentration in human bodies. Examples are liver, mushrooms, shellfish, mussels, cocoa powder, and dried seaweed. An important distribution route is the circulatory system whereas blood vessels are considered to be main stream organs of cadmium toxicity. Chronic inhalation exposure to cadmium particulates is generally associated with changes in pulmonary function and chest radiographs that are consistent with emphysema [93]. Workplace exposure to airborne cadmium particulates has been associated with decreases in olfactory function [94]. Several epidemiologic studies have documented an association of chronic low-level cadmium exposure with decreases in bone mineral density and osteoporosis [95–97].

Exposure to cadmium is commonly determined by measuring cadmium levels in blood or urine. Blood cadmium reflects recent cadmium exposure (e.g., from smoking). Cadmium in urine (usually adjusted for dilution by calculating the cadmium/creatinine ratio) indicates accumulation, or kidney burden of cadmium [98, 99]. It is estimated that about 2.3% of the US population has elevated levels of urine cadmium (>2 μg/g creatinine), a marker of chronic exposure and body burden [100]. Blood and urine cadmium levels are typically higher in cigarette smokers, intermediate in former smokers, and lower in nonsmokers [100, 101]. Because of continuing use of cadmium in industrial applications, the environmental contamination and human exposure to cadmium have dramatically increased during the past century [102].

Molecular Mechanisms of Toxicity and Carcinogenicity

Cadmium is a severe pulmonary and gastrointestinal irritant, which can be fatal if inhaled or ingested. After acute ingestion, symptoms such as abdominal pain, burning sensation, nausea, vomiting, salivation, muscle cramps, vertigo, shock, loss of consciousness, and convulsions usually appear within 15–30 min [103]. Acute cadmium ingestion can also cause gastrointestinal tract erosion; pulmonary,

hepatic, or renal injury; and coma, depending on the route of poisoning [103, 104]. Chronic exposure to cadmium has a depressive effect on levels of norepinephrine, serotonin, and acetylcholine [105]. Rodent studies have shown that chronic inhalation of cadmium causes pulmonary adenocarcinomas [106, 107]. It can also cause prostatic proliferative lesions including adenocarcinomas, after systemic or direct exposure [108].

Although the mechanisms of cadmium toxicity are poorly understood, it has been speculated that cadmium causes damage to cells primarily through the generation of ROS [109], which causes single-strand DNA damage and disrupts the synthesis of nucleic acids and proteins [110]. Studies using two-dimensional gel electrophoresis have shown that several stress response systems are expressed in response to cadmium exposure, including those for heat shock, oxidative stress, stringent response, cold shock, and SOS [111–113]. *In vitro* studies indicate that cadmium induces cytotoxic effects at the concentrations 0.1 to 10 mM and free radical-dependent DNA damage [114, 115]. *In vivo* studies have shown that cadmium modulates male reproduction in mice model at a concentration of 1 mg/kg body weight [116]. However, cadmium is a weak mutagen when compared with other carcinogenic metals [117]. Previous reports have indicated that cadmium affects signal transduction pathways, inducing inositol polyphosphate formation, increasing cytosolic free calcium levels in various cell types [118], and blocking calcium channels [119, 120]. At lower concentrations (1–100 μM), cadmium binds to proteins; decreases DNA repair [121]; activates protein degradation; upregulates cytokines and proto-oncogenes such as c-*fos*, c-*jun*, and c-*myc* [122]; and induces expression of several genes including metallothioneins [123], heme oxygenases, glutathione *S*-transferases, heat-shock proteins, acute-phase reactants, and DNA polymerase β [124].

Cadmium compounds are classified as human carcinogens by several regulatory agencies. The IARC [89] and the US National Toxicology Program have concluded that there is adequate evidence that cadmium is a human carcinogen. This designation as a human carcinogen is based primarily on repeated findings of an association between occupational cadmium exposure and lung cancer, as well as on very strong rodent data showing the pulmonary system as a target site [89]. Thus, the lung is the most definitively established site of human carcinogenesis from cadmium exposure. Other target tissues of cadmium carcinogenesis in animals include injection sites, adrenals, testes, and the hemopoietic system [89, 106, 107]. In some studies, occupational or environmental cadmium exposure has also been associated with development of cancers of the prostate, kidney, liver, hematopoietic system, and stomach [106, 107]. Carcinogenic metals including arsenic, cadmium, chromium, and nickel have all been associated with DNA damage through base pair mutation, deletion, or oxygen radical attack on DNA [124]. Animal studies have demonstrated reproductive and teratogenic effects. Small epidemiologic studies have noted an inverse relationship between cadmium in cord blood, maternal blood, or maternal urine and birth weight and length at birth [125, 126].

Chromium

Environmental Occurrence, Industrial Production and Use

Chromium (Cr) is a naturally occurring element present in the earth's crust, with oxidation states (or valence states) ranging from chromium (II) to chromium (VI) [127]. Chromium compounds are stable in the trivalent [Cr(III)] form and occur in nature in this state in ores, such as ferrochromite. The hexavalent [Cr(VI)] form is the second most stable state [28]. Elemental chromium [Cr(0)] does not occur naturally. Chromium enters into various environmental matrices (air, water, and soil) from a wide variety of natural and anthropogenic sources with the largest release occurring from industrial establishments. Industries with the largest contribution to chromium release include metal processing, tannery facilities, chromate production, stainless steel welding, and ferrochrome and chrome pigment production. The increase in the environmental concentrations of chromium has been linked to air and wastewater release of chromium, mainly from metallurgical, refractory, and chemical industries. Chromium released into the environment from anthropogenic activity occurs mainly in the hexavalent form [Cr(VI)] [128]. Hexavalent chromium [Cr(VI)] is a toxic industrial pollutant that is classified as human carcinogen by several regulatory and nonregulatory agencies [128–130]. The health hazard associated with exposure to chromium depends on its oxidation state, ranging from the low toxicity of the metal form to the high toxicity of the hexavalent form. All Cr(VI)-containing compounds were once thought to be man-made, with only Cr(III) naturally ubiquitous in air, water, soil, and biological materials. Recently, however, naturally occurring Cr(VI) has been found in ground and surface waters at values exceeding the World Health Organization limit for drinking water of 50 μg of Cr(VI) per liter [131]. Chromium is widely used in numerous industrial processes and, as a result, is a contaminant of many environmental systems [132]. Commercially, chromium compounds are used in industrial welding, chrome plating, dyes and pigments, leather tanning, and wood preservation. Chromium is also used as anticorrosive in cooking systems and boilers [133, 134].

Potential for Human Exposure

It is estimated that more than 300,000 workers are exposed annually to chromium and chromium-containing compounds in the workplace. Occupational exposure has been a major concern because of the high risk of Cr-induced diseases in industrial workers occupationally exposed to Cr(VI) [135]. However, the general human population and some wildlife may also be at risk. It is estimated that 33 tons of total chromium are released annually into the environment [128]. In humans and animals, [Cr(III)] is an essential nutrient that plays a role in glucose, fat, and protein

metabolism by potentiating the action of insulin [5]. The US Occupational Safety and Health Administration (OSHA) recently set a "safe" level of 5 μg/m^3, for an 8-h time-weighted average, even though this revised level may still pose a carcinogenic risk [136]. For the general human population, atmospheric levels range from 1 to 100 ng/cm^3 [137], but can exceed this range in areas that are close to chromium manufacturing.

Non-occupational exposure occurs via ingestion of chromium-containing food and water, whereas occupational exposure occurs via inhalation [138]. Chromium concentrations range between 1 and 3,000 mg/kg in soil, 5–800 μg/L in seawater, and 26 μg/L–5.2 mg/L in rivers and lakes [127]. Chromium content in foods varies greatly and depends on the processing and preparation. In general, most fresh foods typically contain chromium levels ranging from <10 to 1,300 μg/kg. Present day workers in chromium-related industries can be exposed to chromium concentrations two orders of magnitude higher than the general population [128]. Even though the principal route of human exposure to chromium is through inhalation and the lung is the primary target organ, significant human exposure to chromium has also been reported to take place through the skin [139, 140]. For example, the widespread incidence of dermatitis noticed among construction workers is attributed to their exposure to chromium present in cement [140]. Occupational and environmental exposure to Cr(VI)-containing compounds is known to cause multi-organ toxicity such as renal damage, allergy and asthma, and cancer of the respiratory tract in humans [5, 141].

Breathing high levels of Cr(VI) can cause irritation to the lining of the nose and nose ulcers. The main health problems seen in animals following ingestion of Cr(VI) compounds are irritation and ulcers in the stomach and small intestine, anemia, sperm damage, and male reproductive system damage. Cr(III) compounds are much less toxic and do not appear to cause these problems. Some individuals are extremely sensitive to Cr(VI) or Cr(III); allergic reactions consisting of severe redness and swelling of the skin have been noted. An increase in stomach tumors was observed in humans and animals exposed to Cr(VI) in drinking water. Accidental or intentional ingestion of extremely high doses of Cr(VI) compounds by humans has resulted in severe respiratory, cardiovascular, gastrointestinal, hematological, hepatic, renal, and neurological effects as part of the sequelae, leading to death or in patients who survived because of medical treatment [128]. Although the evidence of carcinogenicity of chromium in humans and terrestrial mammals seems strong, the mechanism by which it causes cancer is not completely understood [142].

Molecular Mechanisms of Toxicity and Carcinogenicity

Major factors governing the toxicity of chromium compounds are oxidation state and solubility. Cr(VI) compounds, which are powerful oxidizing agents and thus tend to be irritating and corrosive, appear to be much more toxic systemically than

Cr(III) compounds, given similar amount and solubility [143, 144]. Although the mechanisms of biological interaction are uncertain, the variation in toxicity may be related to the ease with which Cr(VI) can pass through cell membranes and its subsequent intracellular reduction to reactive intermediates. Since Cr(III) is poorly absorbed by any route, the toxicity of chromium is mainly attributable to the Cr(VI) form. It can be absorbed by the lung and gastrointestinal tract and even to a certain extent by intact skin. The reduction of Cr(VI) is considered as being a detoxification process when it occurs at a distance from the target site for toxic or genotoxic effect, while reduction of Cr(VI) may serve to activate chromium toxicity if it takes place in or near the cell nucleus of target organs [145]. If Cr(VI) is reduced to Cr(III) extracellularly, this form of the metal is not readily transported into cells, and so toxicity is not observed. The balance that exists between extracellular Cr(VI) and intracellular Cr(III) is what ultimately dictates the amount and rate at which Cr(VI) can enter cells and impart its toxic effects [132].

Cr(VI) enters many types of cells and, under physiological conditions, can be reduced by hydrogen peroxide (H_2O_2), glutathione (GSH) reductase, ascorbic acid, and GSH to produce reactive intermediates, including Cr(V), Cr(IV), thiyl radicals, hydroxyl radicals, and ultimately, Cr(III). Any of these species could attack DNA, proteins, and membrane lipids, thereby disrupting cellular integrity and functions [146, 147].

Studies with animal models have also reported many harmful effects of Cr(VI) on mammals. Subcutaneous administration of Cr(VI) to rats caused severe progressive proteinuria, urea nitrogen and creatinine, as well as elevation in serum alanine aminotransferase activity and hepatic lipid peroxide formation [148]. Similar studies reported by Gumbleton and Nicholls [149] found that Cr(VI) induced renal damage in rats when administered by single subcutaneous injections. Bagchi *et al.* demonstrated that rats received Cr(VI) orally in water-induced hepatic mitochondrial and microsomal lipid peroxidation as well as enhanced excretion of urinary lipid metabolites including malondialdehyde [150, 151].

Adverse health effects induced by Cr(VI) have also been reported in humans. Epidemiological investigations have reported respiratory cancers in workers occupationally exposed to Cr(VI)-containing compounds [139, 145]. DNA strand breaks in peripheral lymphocytes and lipid peroxidation products in urine observed in chromium-exposed workers also support the evidence of Cr(VI)-induced toxicity to humans [152, 153]. Oxidative damage is considered to be the underlying cause of these genotoxic effects including chromosomal abnormalities [154, 155] and DNA strand breaks [156]. Nevertheless, recent studies indicate a biological relevance of non-oxidative mechanisms in Cr(VI) carcinogenesis [157].

Carcinogenicity appears to be associated with the inhalation of the less soluble/insoluble Cr(VI) compounds. The toxicology of Cr(VI) does not reside with the elemental form. It varies greatly among a wide variety of very different Cr(VI) compounds [158]. Epidemiological evidence strongly points to Cr(VI) as the agent in carcinogenesis. Solubility and other characteristics of chromium, such as size, crystal modification, surface charge, and the ability to be phagocytized might be important in determining cancer risk [133].

Studies in our laboratory have indicated that Cr(VI) is cytotoxic and able to induce DNA-damaging effects such as chromosomal abnormalities [159], DNA strand breaks, DNA fragmentation, and oxidative stress in Sprague–Dawley rats and human liver carcinoma cells [27, 28]. Recently, our laboratory has also demonstrated that Cr(VI) induces biochemical, genotoxic, and histopathologic effects in liver and kidney of goldfish, *Carassius auratus* [160].

Various hypotheses have been proposed to explain the carcinogenicity of chromium and its salts; however, some inherent difficulties exist when discussing metal carcinogenesis. A metal cannot be classified as carcinogenic per se since its different compounds may have different potencies. Because of the multiple chemical exposure in industrial establishments, it is difficult from an epidemiological standpoint to relate the carcinogenic effect to a single compound. Thus, the carcinogenic risk must often be related to a process or to a group of metal compounds rather than to a single substance. Differences in carcinogenic potential are related not only to different chemical forms of the same metal but also to the particle size of the inhaled aerosol and to physical characteristics of the particle such as surface charge and crystal modification [161].

Lead

Environmental Occurrence, Industrial Production and Use

Lead is a naturally occurring bluish-gray metal present in small amounts in the earth's crust. Although lead occurs naturally in the environment, anthropogenic activities such as fossil fuels burning, mining, and manufacturing contribute to the release of high concentrations. Lead has many different industrial, agricultural, and domestic applications. It is currently used in the production of lead–acid batteries, ammunitions, metal products (solder and pipes), and devices to shield X-rays. An estimated 1.52 million metric tons of lead were used for various industrial applications in the United States in 2004. Of that amount, lead–acid batteries production accounted for 83%, and the remaining usage covered a range of products such as ammunitions (3.5%), oxides for paint, glass, pigments and chemicals (2.6%), and sheet lead (1.7%) [162, 163].

In recent years, the industrial use of lead has been significantly reduced from paints and ceramic products, caulking, and pipe solder [164]. Despite this progress, it has been reported that among 16.4 million US homes with more than one child younger than 6 years per household, 25% of homes still had significant amounts of lead-contaminated deteriorated paint, dust, or adjacent bare soil [165]. Lead in dust and soil often recontaminates cleaned houses [166] and contributes to elevating blood lead concentrations in children who play on bare, contaminated soil [167]. Today, the largest source of lead poisoning in children comes from dust and chips from deteriorating lead paint on interior surfaces [168]. Children who live in homes

with deteriorating lead paint can achieve blood lead concentrations of 20 μg/dL or greater [169].

Potential for Human Exposure

Exposure to lead occurs mainly via inhalation of lead-contaminated dust particles or aerosols and ingestion of lead-contaminated food, water, and paints [170, 171]. Adults absorb 35–50% of lead through drinking water, and the absorption rate for children may be greater than 50%. Lead absorption is influenced by factors such as age and physiological status. In the human body, the greatest percentage of lead is taken into the kidney, followed by the liver and the other soft tissues such as heart and brain; however, the lead in the skeleton represents the major body fraction [172]. The nervous system is the most vulnerable target of lead poisoning. Headache, poor attention spam, irritability, loss of memory, and dullness are the early symptoms of the effects of lead exposure on the central nervous system [167, 170].

Since the late 1970s, lead exposure has decreased significantly as a result of multiple efforts including the elimination of lead in gasoline and the reduction of lead levels in residential paints, food and drink cans, and plumbing systems [170, 171]. Several federal programs implemented by state and local health governments have not only focused on banning lead in gasoline, paint, and soldered cans but have also supported screening programs for lead poisoning in children and lead abatement in housing [164]. Despite the progress in these programs, human exposure to lead remains a serious health problem [173, 174]. Lead is the most systemic toxicant that affects several organs in the body including the kidneys, liver, central nervous system, hematopoietic system, endocrine system, and reproductive system [170].

Lead exposure usually results from lead in deteriorating household paints, lead in the workplace, lead in crystals and ceramic containers that leaches into water and food, lead use in hobbies, and lead use in some traditional medicines and cosmetics [164, 171]. Several studies conducted by the National Health and Nutrition Examination surveys (NHANES) have measured blood lead levels in the US populations and have assessed the magnitude of lead exposure by age, gender, race, income, and degree of urbanization [173]. Although the results of these surveys have demonstrated a general decline in blood lead levels since the 1970s, they have also shown that large populations of children continue to have elevated blood lead levels (>10 μg/dL). Hence, lead poisoning remains one of the most common pediatric health problems in the United States today [164, 170, 171, 173–176]. Exposure to lead is of special concern among women particularly during pregnancy. Lead absorbed by the pregnant mother is readily transferred to the developing fetus [177]. Human evidence corroborates animal findings [178], linking prenatal exposure to lead with reduced birth weight and preterm delivery [179], and with neurodevelopmental abnormalities in offspring [180].

Molecular Mechanisms of Toxicity and Carcinogenicity

There are many published studies that have documented the adverse effects of lead in children and the adult population. In children, these studies have shown an association between blood level poisoning and diminished intelligence, lower intelligence quotient—IQ, delayed or impaired neurobehavioral development, decreased hearing acuity, speech and language handicaps, growth retardation, poor attention span, and antisocial and diligent behaviors [175, 176, 181, 182]. In the adult population, reproductive effects, such as decreased sperm count in men and spontaneous abortions in women, have been associated with high lead exposure [183, 184]. Acute exposure to lead induces brain damage, kidney damage, and gastrointestinal diseases, while chronic exposure may cause adverse effects on the blood, central nervous system, blood pressure, kidneys, and vitamin D metabolism [170, 171, 175, 176, 181–184].

One of the major mechanisms by which lead exerts its toxic effect is through biochemical processes that include lead's ability to inhibit or mimic the actions of calcium and to interact with proteins [170]. Within the skeleton, lead is incorporated into the mineral in place of calcium. Lead binds to biological molecules and thereby interfering with their function by a number of mechanisms. Lead binds to sulfhydryl and amide groups of enzymes, altering their configuration and diminishing their activities. Lead may also compete with essential metallic cations for binding sites, inhibiting enzyme activity, or altering the transport of essential cations such as calcium [185]. Many investigators have demonstrated that lead intoxication induces a cellular damage mediated by the formation of ROS [186]. In addition, Jiun and Hsien [187] demonstrated that the levels of malondialdehyde (MDA) in blood strongly correlate with lead concentration in the blood of exposed workers. Other studies showed that the activities of antioxidant enzymes, including superoxide dismutase (SOD) and glutathione peroxidase in erythrocytes of workers exposed to lead, are remarkably higher than that in non-exposed workers [188]. A series of recent studies in our laboratory demonstrated that lead-induced toxicity and apoptosis in human cancer cells involved several cellular and molecular processes including induction of cell death and oxidative stress [29, 189], transcriptional activation of stress genes [30], DNA damage [29], externalization of phosphatidylserine, and activation of caspase 3 [190].

A large body of research has indicated that lead acts by interfering with calcium-dependent processes related to neuronal signaling and intracellular signal transduction. Lead perturbs intracellular calcium cycling, altering releasability of organelle stores, such as endoplasmic reticulum and mitochondria [191, 192]. In some cases, lead inhibits calcium-dependent events, including calcium-dependent release of several neurotransmitters and receptor-coupled ionophores in glutamatergic neurons [193]. In other cases, lead appears to augment calcium-dependent events, such as protein kinase C and calmodulin [191, 194].

Experimental studies have indicated that lead is potentially carcinogenic, inducing renal tumors in rats and mice [195, 196], and is therefore considered by the

IARC as a probable human carcinogen [197]. Lead exposure is also known to induce gene mutations and sister chromatid exchanges [198, 199], morphological transformations in cultured rodent cells [200], and to enhance anchorage independence in diploid human fibroblasts [123]. *In vitro* and *in vivo* studies indicated that lead compounds cause genetic damage through various indirect mechanisms that include inhibition of DNA synthesis and repair, oxidative damage, and interaction with DNA-binding proteins and tumor suppressor proteins. Studies by Roy and his group showed that lead acetate induced mutagenicity at a toxic dose at the *Escherichia coli gpt* locus transfected to V79 cells [201]. They also reported that toxic doses of lead acetate and lead nitrate induced DNA breaks at the *E. coli gpt* locus transfected to V79 cells [201]. Another study by Wise and his collaborators found no evidence for direct genotoxic or DNA-damaging effects of lead except for lead chromate. They pointed out that the genotoxicity may be due to hexavalent chromate rather than lead [202].

Mercury

Environmental Occurrence, Industrial Production and Use

Mercury is a heavy metal belonging to the transition element series of the periodic table. It is unique in that it exists or is found in nature in three forms (elemental, inorganic, and organic), with each having its own profile of toxicity [203]. At room temperature, elemental mercury exists as a liquid which has a high vapor pressure and is released into the environment as mercury vapor. Mercury also exists as a cation with oxidation states of +1 (mercurous) or +2 (mercuric) [204]. Methylmercury is the most frequently encountered compound of the organic form found in the environment and is formed as a result of the methylation of inorganic (mercuric) forms of mercury by microorganisms found in soil and water [205].

Mercury is a widespread environmental toxicant and pollutant which induces severe alterations in the body tissues and causes a wide range of adverse health effects [206]. Both humans and animals are exposed to various chemical forms of mercury in the environment. These include elemental mercury vapor (Hg^0), inorganic mercurous (Hg^{1+}), mercuric (Hg^{2+}), and the organic mercury compounds [207]. Because mercury is ubiquitous in the environment, humans, plants, and animals are all unable to avoid exposure to some form of mercury [208].

Mercury is utilized in the electrical industry (switches, thermostats, batteries), dentistry (dental amalgams), and numerous industrial processes including the production of caustic soda, in nuclear reactors, as antifungal agents for wood processing, as a solvent for reactive and precious metal, and as a preservative of pharmaceutical products [209]. The industrial demand for mercury peaked in 1964 and began to sharply decline between 1980 and 1994 as a result of federal bans on mercury additives in paints, pesticides, and the reduction of its use in batteries [210].

Potential for Human Exposure

Humans are exposed to all forms of mercury through accidents, environmental pollution, food contamination, dental care, preventive medical practices, industrial and agricultural operations, and occupational operations [206]. The major sources of chronic low-level mercury exposure are dental amalgams and fish consumption. Mercury enters water as a natural process of off-gassing from the earth's crust and also through industrial pollution [205]. Algae and bacteria methylate the mercury entering the waterways. Methyl mercury then makes its way through the food chain into fish, shellfish, and eventually into humans [211].

The two most highly absorbed species are elemental mercury (Hg^0) and methyl mercury (MeHg). Dental amalgams contain over 50% elemental mercury [207]. The elemental vapor is highly lipophilic and is effectively absorbed through the lungs and tissues lining the mouth. After Hg^0 enters the blood, it rapidly passes through cell membranes, which include both the blood–brain barrier and the placental barrier [204]. Once it gains entry into the cell, Hg^0 is oxidized and becomes highly reactive Hg^{2+}. Methyl mercury derived from eating fish is readily absorbed in the gastrointestinal tract and, because of its lipid solubility, can easily cross both the placental and blood–brain barriers. Once mercury is absorbed, it has a very low excretion rate. A major proportion of what is absorbed accumulates in the kidneys, neurological tissue, and the liver. All forms of mercury are toxic, and their effects include gastrointestinal toxicity, neurotoxicity, and nephrotoxicity [209].

Molecular Mechanisms of Toxicity and Carcinogenicity

The molecular mechanisms of toxicity of mercury are based on its chemical activity and biological features which suggest that oxidative stress is involved in its toxicity [212]. Through oxidative stress, mercury has shown mechanisms of sulfhydryl reactivity. Once in the cell, both Hg^{2+} and MeHg form covalent bonds with cysteine residues of proteins and deplete cellular antioxidants. Antioxidant enzymes serve as a line of cellular defense against mercury compounds [213]. The interaction of mercury compounds suggests the production of oxidative damage through the accumulation of ROS which would normally be eliminated by cellular antioxidants.

In eukaryotic organisms, the primary site for the production of ROS occurs in the mitochondria through normal metabolism [214]. Inorganic mercury has been reported to increase the production of these ROS by causing defects in oxidative phosphorylation and electron transport at the ubiquinone–cytochrome b_5 step [215]. Through the acceleration of the rate of electron transfer in the electron transport chain in the mitochondria, mercury induces the premature shedding of electrons to molecular oxygen which causes an increase in the generation of ROS [216].

Oxidative stress appears to also have an effect on calcium homeostasis. The role of calcium in the activation of proteases, endonucleases, and phospholipases is well

established. The activation of phospholipase A_2 has been shown to result in an increase in ROS through the increase generation of arachidonic acid. Arachidonic acid has also been shown to be an important target of ROS [217]. Both organic and inorganic mercury have been shown to alter calcium homeostasis but through different mechanisms. Organic mercury compounds (MeHg) are believed to increase intracellular calcium by accelerating the influx of calcium from the extracellular medium and mobilizing intracellular stores, while inorganic mercury (Hg^{2+}) compounds increase intracellular calcium stores only through the influx of calcium from the extracellular medium [218]. Mercury compounds have also been shown to induce increased levels of MDA in the livers, kidneys, lungs, and testes of rats treated with $HgCl_2$ [219]. This increase in concentration was shown to correlate with the severity of hepatotoxicity and nephrotoxicity [216]. $HgCl_2$-induced lipid peroxidation was shown to be significantly reduced by antioxidant pretreatment with selenium. Selenium has been shown to achieve this protective effect through direct binding to mercury or serving as a cofactor for glutathione peroxidase and facilitating its ability to scavenge ROS [220]. Vitamin E has also been reported to protect against $HgCl_2$-induced lipid peroxidation in the liver [221].

Metal-induced carcinogenicity has been a research subject of great public health interest. Generally, carcinogenesis is considered to have three stages including initiation, promotion, and progression and metastasis. Although mutations of DNA, which can activate oncogenesis or inhibit tumor suppression, were traditionally thought to be crucial factors for the initiation of carcinogenesis, recent studies have demonstrated that other molecular events, such as transcription activation, signal transduction, oncogene amplification, and recombination, also constitute significant contributing factors [222, 223]. Studies have shown that mercury and other toxic metals affect cellular organelles and adversely affect their biologic functions [222, 224]. Accumulating evidence also suggests that ROS play a major role in the mediation of metal-induced cellular responses and carcinogenesis [225–227].

The connection between mercury exposure and carcinogenesis is very controversial. While some studies have confirmed its genotoxic potential, others have not shown an association between mercury exposure and genotoxic damage [226]. In studies implicating mercury as a genotoxic agent, oxidative stress has been described as the molecular mechanism of toxicity. Hence, mercury has been shown to induce the formation of ROS known to cause DNA damage in cells, a process which can lead to the initiation of carcinogenic processes [213, 228]. The direct action of these free radicals on nucleic acids may generate genetic mutations. Although mercury-containing compounds are not mutagenic in bacterial assays, inorganic mercury has been shown to induce mutational events in eukaryotic cell lines with doses as low as 0.5 μM [229]. These free radicals may also induce conformational changes in proteins that are responsible for DNA repair, mitotic spindle, and chromosomal segregation [213]. To combat these effects, cells have antioxidant mechanisms that work to correct and avoid the formation of ROS (free radicals) in excess. These antioxidant mechanisms involve low molecular weight compounds such as vitamins C and E, melatonin, glutathione, superoxide

dismutase, catalase, glutathione peroxidase, and glutathione reductase that protect the cells by chelating mercury and reducing its oxidative stress potential [230].

Glutathione levels in human populations exposed to methylmercury intoxication by eating contaminated fish have been shown to be higher than normal [231]. These studies were also able to confirm a direct and positive correlation between mercury and glutathione levels in blood. They also confirmed an increased mitotic index and polyploidal aberrations associated with mercury exposure [231]. Epidemiological studies have demonstrated that enzymatic activity was altered in populations exposed to mercury, producing genotoxic alterations and suggesting that both chronic and relatively low-level mercury exposures may inhibit enzyme activity and induce oxidative stress in the cells [232]. There is no doubt that the connection between mercury exposure and carcinogenesis is very controversial. However, *in vitro* studies suggest that the susceptibility to DNA damage exists as a result of cellular exposure to mercury. These studies also indicate that mercury-induced toxicity and carcinogenicity may be cell, organ, and/or species specific.

Prospects

A comprehensive analysis of published data indicates that heavy metals such as arsenic, cadmium, chromium, lead, and mercury occur naturally. However, anthropogenic activities contribute significantly to environmental contamination. These metals are systemic toxicants known to induce adverse health effects in humans, including cardiovascular diseases, developmental abnormalities, neurologic and neurobehavioral disorders, diabetes, hearing loss, hematologic and immunologic disorders, and various types of cancer. The main pathways of exposure include ingestion, inhalation, and dermal contact. The severity of adverse health effects is related to the type of heavy metal and its chemical form and is also time and dose dependent. Among many other factors, speciation plays a key role in metal toxicokinetics and toxicodynamics and is highly influenced by factors such as valence state, particle size, solubility, biotransformation, and chemical form. Several studies have shown that toxic metal exposure causes long-term health problems in human populations. Although the acute and chronic effects are known for some metals, little is known about the health impact of mixtures of toxic elements. Recent reports have pointed out that these toxic elements may interfere metabolically with nutritionally essential metals such as iron, calcium, copper, and zinc [233, 234]. However, the literature is scarce regarding the combined toxicity of heavy metals. Simultaneous exposure to multiple heavy metals may produce a toxic effect that is additive, antagonistic, or synergistic.

A recent review of a number of individual studies that addressed metals interactions reported that co-exposure to metal/metalloid mixtures of arsenic, lead, and cadmium produced more severe effects at both relatively high-dose and low-dose levels in a biomarker-specific manner [235]. These effects were found to be mediated by dose, duration of exposure, and genetic factors. Also, human co-exposure to

cadmium and inorganic arsenic resulted in a more pronounced renal damage than exposure to each of the elements alone [236]. In many areas of metal pollution, chronic low-dose exposure to multiple elements is a major public health concern. Elucidating the mechanistic basis of heavy metal interactions is essential for health risk assessment and management of chemical mixtures. Hence, research is needed to further elucidate the molecular mechanisms and public health impact associated with human exposure to mixtures of toxic metals.

Acknowledgments This research was supported by the National Institutes of Health RCMI Grant No. 2G12RR013459 and in part by the National Oceanic and Atmospheric Administration ECSC Grant No. NA06OAR4810164 and Subcontract No. 000953.

References

1. Fergusson JE (1990) The heavy elements: chemistry, environmental impact and health effects. Pergamon, Oxford
2. Duffus JH (2002) Heavy metals—a meaningless term? Pure Appl Chem 74:793–807
3. Bradl H (2002) Heavy metals in the environment: origin, interaction and remediation, vol 6. Academic, London
4. He ZL, Yang XE, Stoffella PJ (2005) Trace elements in agroecosystems and impacts on the environment. J Trace Elem Med Biol 19:125–140
5. Goyer RA (2001) Toxic effects of metals. In: Klaassen CD (ed) Cassarett and Doull's toxicology: the basic science of poisons. McGraw-Hill, New York, NY, pp 811–867
6. Herawati N, Suzuki S, Hayashi K, Rivai IF, Koyoma H (2000) Cadmium, copper and zinc levels in rice and soil of Japan, Indonesia and China by soil type. Bull Env Contam Toxicol 64:33–39
7. Shallari S, Schwartz C, Hasko A, Morel JL (1998) Heavy metals in soils and plants of serpentine and industrial sites of Albania. Sci Total Environ 209:133–142
8. Nriagu JO (1989) A global assessment of natural sources of atmospheric trace metals. Nature 338:47–49
9. Arruti A, Fernández-Olmo I, Irabien A (2010) Evaluation of the contribution of local sources to trace metals levels in urban PM2.5 and PM10 in the Cantabria region (Northern Spain). J Environ Monit 12:1451–1458
10. Sträter E, Westbeld A, Klemm O (2010) Pollution in coastal fog at Alto Patache, Northern Chile. Environ Sci Pollut Res Int 17:1563–1573
11. Pacyna JM (1996) Monitoring and assessment of metal contaminants in the air. In: Chang LW, Magos L, Suzuli T (eds) Toxicology of metals. CRC, Boca Raton, FL, pp 9–28
12. WHO/FAO/IAEA (1996) Trace elements in human nutrition and health. World Health Organization, Geneva
13. Kabata-Pendia A (2001) Trace elements in soils and plants, 3rd edn. CRC, Boca Raton, FL
14. Hamelink JL, Landrum PF, Harold BL, William BH (1994) Bioavailability: physical, chemical, and biological interactions. CRC, Boca Raton, FL
15. Verkleji JAS (1993) The effects of heavy metals stress on higher plants and their use as biomonitors. In: Markert B (ed) Plant as bioindicators: indicators of heavy metals in the terrestrial environment. VCH, New York, NY, pp 415–424
16. Stern BR (2010) Essentiality and toxicity in copper health risk assessment: overview, update and regulatory considerations. Toxicol Environ Health A 73:114–127
17. Harvey LJ, McArdle HJ (2008) Biomarkers of copper status: a brief update. Br J Nutr 99: S10–S13

18. ATSDR (2002) Toxicological profile for copper. Centers for Disease Control, Agency for Toxic Substances and Disease Registry, Atlanta, GA
19. Tchounwou P, Newsome C, Williams J, Glass K (2008) Copper-induced cytotoxicity and transcriptional activation of stress genes in human liver carcinoma cells. Metal Ions Biol Med 10:285–290
20. Chang LW, Magos L, Suzuki T (1996) Toxicology of metals. CRC, Boca Raton, FL
21. Wang S, Shi X (2001) Molecular mechanisms of metal toxicity and carcinogenesis. Mol Cell Biochem 222:3–9
22. Beyersmann D, Hartwig A (2008) Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. Arch Toxicol 82:493–512
23. Yedjou CG, Tchounwou PB (2006) Oxidative stress in human leukemia cells (HL-60), human liver carcinoma cells ($HepG_2$) and human Jerkat-T cells exposed to arsenic trioxide. Metal Ions Biol Med 9:298–303
24. Yedjou GC, Tchounwou PB (2007) *In vitro* cytotoxic and genotoxic effects of arsenic trioxide on human leukemia cells using the MTT and alkaline single cell gel electrophoresis (comet) assays. Mol Cell Biochem 301:123–130
25. Tchounwou PB, Centeno JA, Patlolla AK (2004) Arsenic toxicity, mutagenesis and carcinogenesis—a health risk assessment and management approach. Mol Cell Biochem 255:47–55
26. Tchounwou PB, Ishaque A, Schneider J (2001) Cytotoxicity and transcriptional activation of stress genes in human liver carcinoma cells ($HepG_2$) exposed to cadmium chloride. Mol Cell Biochem 222:21–28
27. Patlolla A, Barnes C, Field J, Hackett D, Tchounwou PB (2009) Potassium dichromate-induced cytotoxicity, genotoxicity and oxidative stress in human liver carcinoma ($HepG_2$) cells. Int J Environ Res Public Health 6:643–653
28. Patlolla A, Barnes C, Yedjou C, Velma V, Tchounwou PB (2009) Oxidative stress, DNA damage and antioxidant enzyme activity induced by hexavalent chromium in Sprague Dawley rats. Environ Toxicol 24:66–73
29. Yedjou GC, Tchounwou PB (2008) *N*-acetyl-cysteine affords protection against lead-induced cytotoxicity and oxidative stress in human liver carcinoma ($HepG_2$) cells. Int J Environ Res Public Health 4:132–137
30. Tchounwou PB, Yedjou CG, Foxx D, Ishaque A, Shen E (2004) Lead-induced cytotoxicity and transcriptional activation of stress genes in human liver carcinoma cells (HepG2). Mol Cell Biochem 255:161–170
31. Sutton DJ, Tchounwou PB (2007) Mercury induces the externalization of phosphatidylserine in human proximal tubule (HK-2) cells. Int J Environ Res Public Health 4:138–144
32. Sutton D, Tchounwou PB, Ninashvili N, Shen E (2002) Mercury induces cytotoxicity, and transcriptionally activates stress genes in human liver carcinoma cells. Int J Mol Sci 3: 965–984
33. ATSDR (2000) Toxicological profile for arsenic TP-92/09. Center for Disease Control, Agency for Toxic Substances and Disease Registry, Atlanta, GA
34. Tchounwou PB, Wilson B, Ishaque A (1999) Important considerations in the development of public health advisories for arsenic and arsenic-containing compounds in drinking water. Rev Environ Health 14:211–229
35. Centeno JA, Tchounwou PB, Patlolla AK, Mullick FG, Murakat L, Meza E, Gibb H, Longfellow D, Yedjou CG (2005) Environmental pathology and health effects of arsenic poisoning: a critical review. In: Naidu R, Smith E, Smith J, Bhattacharya P (eds) Managing arsenic in the environment: from soil to human health. CSIRO, Adelaide
36. Rousselot P, Laboume S, Marolleau JP, Larghero T, Noguera ML, Brouet JC, Fermand JP (1999) Arsenic trioxide and melarsoprol induce apoptosis in plasma cell lines and in plasma cells from myeloma patients. Cancer Res 59:1041–1048
37. NRCC (1978) Effects of arsenic in the environment. National Research Council of Canada, Ottawa, pp 1–349
38. Morton WE, Dunnette DA (1994) Health effects of environmental arsenic. In: Nriagu JO (ed) Arsenic in the environment part II: human health and ecosystem effects. Wiley, New York, NY, pp 17–34

39. National Research Council (2001) Arsenic in drinking water. 2001 Update. Online available at http://www.nap.edu/books/0309076293/ html/
40. Tchounwou PB, Centeno JA (2008) Toxicologic pathology. In: Gad SC (ed) Handbook of pre-clinical development. Wiley, New York, NY, pp 551–580
41. Tchounwou PB, Patlolla AK, Centeno JA (2003) Carcinogenic and systemic health effects associated with arsenic exposure—a critical review. Toxicol Pathol 31:575–588
42. Tchounwou PB, Wilson BA, Abdelgnani AA, Ishaque AB, Patlolla AK (2002) Differential cytotoxicity and gene expression in human liver carcinoma ($HepG_2$) cells exposed to arsenic trioxide and monosodium acid methanearsonate (MSMA). Int J Mol Sci 3:1117–1132
43. Yedjou GC, Moore P, Tchounwou PB (2006) Dose and time dependent response of human leukemia (HL-60) cells to arsenic trioxide. Int J Environ Res Public Health 3:136–140
44. Chappell W, Beck B, Brown K, North D, Thornton I, Chaney R, Cothern R, Cothern CR, North DW, Irgolic K, Thornton I, Tsongas T (1997) Inorganic arsenic: a need and an opportunity to improve risk assessment. Environ Health Perspect 105:1060–1067
45. Centeno JA, Gray MA, Mullick FG, Tchounwou PB, Tseng C (2005) Arsenic in drinking water and health issues. In: Moore TA, Black A, Centeno JA, Harding JS, Trumm DA (eds) Metal contaminants in New Zealand. Resolution, New Zealand, pp 195–219
46. Abernathy CO, Liu YP, Longfellow D, Aposhian HV, Beck B, Fowler B, Goyer R, Menzer R, Rossman T, Thompson C, Waalkes R (1999) Arsenic: health effects, mechanisms of actions and research issues. Environ Health Perspect 107:593–597
47. Hughes MF (2002) Arsenic toxicity and potential mechanisms of action. Toxicol Lett 133:1–16
48. Wang Z, Rossman TG (1996) The carcinogenicity of arsenic. In: Chang LW, Magos L, Suzuki T (eds) Toxicology of metals, vol 1. CRC, Boca Raton, FL, pp 221–229
49. Belton JC, Benson NC, Hanna ML, Taylor RT (1985) Growth inhibition and cytotoxic effects of three arsenic compounds on cultured Chinese hamster ovary cells. J Environ Sci Health A 20:37–72
50. Li JH, Rossman TC (1989) Inhibition of DNA ligase activity by arsenite: a possible mechanism of its comutagenesis. Mol Toxicol 2:1–9
51. Jha AN, Noditi M, Nilsson R, Natarajan AT (1992) Genotoxic effects of sodium arsenite on human cells. Mutat Res 284:215–221
52. Hartmann A, Speit G (1994) Comparative investigations of the genotoxic effects of metals in the single cell gel assay and the sister-chromatid exchange test. Environ Mol Mutagen 23:299–305
53. Patlolla A, Tchounwou PB (2005) Cytogenetic evaluation of arsenic trioxide toxicity in Sprague-Dawley rats. Mutat Res—Genet Toxicol Environ Mutagen 587:126–133
54. Basu A, Mahata J, Gupta S, Giri AK (2001) Genetic toxicology of a paradoxical human carcinogen, arsenic: a review. Mutat Res 488:171–194
55. Landolph JR (1989) Molecular and cellular mechanisms of transformation of C3H/10T1/2C18 and diploid human fibroblasts by unique carcinogenic, non-mutagenic metal compounds. a review. Biol Trace Elem Res 21:459–467
56. Takahashi M, Barrett JC, Tsutsui T (2002) Transformation by inorganic arsenic compounds of normal Syrian hamster embryo cells into a neoplastic state in which they become anchorage-independent and cause tumors in newborn hamsters. Int J Cancer 99:629–634
57. Anderson D, Yu TW, Phillips BJ, Schmezer P (1994) The effect of various antioxidants and other modifying agents on oxygen radical-generated DNA damage in human lymphocytes in the comet assay. Mutat Res 307:261–271
58. Saleha Banu B, Danadevi K, Jamil K, Ahuja YR, Visweswara Rao K, Ishap M (2001) *In vivo* genotoxic effect of arsenic trioxide in mice using comet assay. Toxicology 162:171–177
59. Barrett JC, Lamb PW, Wang TC, Lee TC (1989) Mechanisms of arsenic-induced cell transformation. Biol Trace Elem Res 21:421–429
60. Tchounwou PB, Yedjou CG, Dorsey WC (2003) Arsenic trioxide-induced transcriptional activation and expression of stress genes in human liver carcinoma cells ($HepG_2$). Cell Mol Biol 49:1071–1079

61. Zhao CQ, Young MR, Diwan BA, Coogan TP, Waalkes MP (1997) Association of arsenic-induced malignant transformation with DNA hypomethylation and aberrant gene expression. Proc Natl Acad Sci USA 94:10907–10912
62. Liu Y, Guyton KZ, Gorospe M, Xu Q, Lee JC, Holbrook NJ (1996) Differential activation of ERK, JNK/SAPK and P38/CSBP/RK map kinase family members during the cellular response to arsenite. Free Radic Biol Med 21:771–781
63. Ludwig S, Hoffmeyer A, Goebeler M, Kilian K, Hafner H, Neufeld B, Han J, Rapp UR (1998) The stress inducer arsenite activates mitogen-activated protein kinases extracellular signal-regulated kinases 1 and 2 via a MAPK kinase 6/p38-dependent pathway. J Biol Chem 273:1917–1922
64. Trouba KJ, Wauson EM, Vorce RL (2000) Sodium arsenite-induced dysregulation of proteins involved in proliferative signaling. Toxicol Appl Pharmacol 164:161–170
65. Vogt BL, Rossman TG (2001) Effects of arsenite on p53, p21 and cyclin D expression in normal human fibroblasts—a possible mechanism for arsenite's comutagenicity. Mutat Res 478:159–168
66. Chen NY, Ma WY, Huang C, Ding M, Dong Z (2000) Activation of PKC is required for arsenite-induced signal transduction. J Environ Pathol Toxicol Oncol 19:297–306
67. Porter AC, Fanger GR, Vaillancourt RR (1999) Signal transduction pathways regulated by arsenate and arsenite. Oncogene 18:7794–7802
68. Soignet SL, Frankel SR, Douer D, Tallman MS, Kantarjian H, Calleja E, Stone RM, Kalaycio M, Scheinberg DA, Steinherz P, Sievers EL, Coutré S, Dahlberg S, Ellison R, Warrell RP Jr (2001) United States multicenter study of arsenic trioxide in relapsed acute promyelocytic leukemia. J Clin Oncol 19:3852–3860
69. Murgo AJ (2001) Clinical trials of arsenic trioxide in hematologic and solid tumors: overview of the National Cancer Institute Cooperative Research and Development Studies. Oncologist 6:22–28
70. Puccetti ES, Guller S, Orleth A, Bruggenolte N, Hoelzer D, Ottmann OG, Ruthardt M (2000) BCR-ABL mediates arsenic trioxide-induced apoptosis independently of its aberrant kinase activity. Cancer Res 60:3409–3413
71. Seol JG, Park WH, Kim ES, Jung CW, Hyun JM, Kim BK, Lee YY (1999) Effect of arsenic trioxide on cell cycle arrest in head and neck cancer cell-line PCI-1. Biochem Biophys Res Commun 265:400–404
72. Alemany M, Levin J (2000) The effects of arsenic trioxide on human megakaryocytic leukemia cell lines with a comparison of its effects on other cell lineages. Leuk Lymphoma 38:153–163
73. Deaglio S, Canella D, Baj G, Arnulfo A, Waxman S, Malavasi F (2001) Evidence of an immunologic mechanism behind the therapeutic effects of arsenic trioxide on myeloma cells. Leuk Res 25:237–239
74. Tully DB, Collins BJ, Overstreet JD, Smith CS, Dinse GE, Mumtaz MM, Chapin RE (2000) Effects of arsenic, cadmium, chromium and lead on gene expression regulated by a battery of 13 different promoters in recombinant $HepG_2$ cells. Toxicol Appl Pharmacol 168:79–90
75. Lu T, Liu J, LeCluyse EL, Zhou YS, Cheng ML, Waalkes MP (2001) Application of cDNA microarray to the study of arsenic-induced liver diseases in the population of Guizhou, China. Toxicol Sci 59:185–192
76. Harris CC (1991) Chemical and physical carcinogenesis: advances and perspectives. Cancer Res 51:5023s–5044s
77. Graham-Evans B, Colhy HHP, Yu H, Tchounwou PB (2004) Arsenic-induced genotoxic and cytotoxic effects in human keratinocytes, melanocytes, and dendritic cells. Int J Environ Res Public Health 1:83–89
78. Stevens JJ, Graham B, Walker AM, Tchounwou PB, Rogers C (2010) The effects of arsenic trioxide on DNA synthesis and genotoxicity in human colon cancer cells. Int J Environ Res Public Health 7:2018–2032
79. Walker AM, Stevens JJ, Ndebele K, Tchounwou PB (2010) Arsenic trioxide modulates DNA synthesis and apoptosis in lung carcinoma cells. Int J Environ Res Public Health 7: 1996–2007

80. Yedjou CG, Tchounwou PB (2009) Modulation of p53, *c-fos*, RARE, cyclin A and cyclin D1 expression in human leukemia (HL-60) cells exposed to arsenic trioxide. Mol Cell Biochem 331:207–214
81. Yedjou C, Sutton LM, Tchounwou PB (2008) Genotoxic mechanisms of arsenic trioxide effect in human Jurkat T-lymphoma cells. Metal Ions Biol Med 10:495–499
82. Brown E, Yedjou C, Tchounwou PB (2008) Cytotoxicty and oxidative stress in human liver carcinoma cells exposed to arsenic trioxide. Metal Ions Biol Med 10:583–587
83. Yedjou CG, Thuisseu L, Tchounwou C, Gomes M, Howard C, Tchounwou PB (2009) Ascorbic acid potentiation of arsenic trioxide anticancer activity against acute promyelocytic leukemia. Arch Drug Inf 2:59–65
84. Yedjou C, Rogers C, Brown E, Tchounwou P (2008) Differential effect of ascorbic acid and *N*-acetyl-cysteine on arsenic trioxide-mediated oxidative stress in human leukemia (HL-60) cells. J Biochem Mol Toxicol 22:85–92
85. Miller WH, Schipper HM, Lee JS, Singer J, Waxman S (2002) Mechanisms of action of arsenic trioxide. Cancer Res 62:3893–3903
86. Gesamp (1987) IMO/FAO/UNESCO/WMO/WHO/IAEA/UN/UNEP joint group of experts on the scientific aspects of marine pollution: report of the seventeenth session, reports and studies No. 31. World Health Organization, Geneva, Switzerland
87. Wilson DN (1988) Cadmium—market trends and influences. In: Cadmium association (ed) Cadmium 87, Proceedings of the 6th International Cadmium Conference, London, pp 9–16
88. U.S. EPA (2006) Cadmium compounds. U.S. Environmental Protection Agency. Online available at http://www.epa.gov/ttnatw01/hlthef/cadmium.html
89. IARC (1993) Cadmium. International Agency for Research on Cancer, Lyon
90. Paschal DC, Burt V, Caudill SP, Gunter EW, Pirkle JL, Sampson EJ, Miller DT, Jackson RJ (2000) Exposure of the U.S. population aged 6 years and older to cadmium: 1988–1994. Arch Environ Contam Toxicol 38:377–383
91. ATSDR (2008) Draft toxicological profile for cadmium. Agency for Toxic Substances and Disease Registry, Atlanta, GA
92. Satarug S, Baker JR, Urbenjapol S, Haswell-Elkins M, Reilly PE, Williams DJ, Moore MR (2003) A global perspective on cadmium pollution and toxicity in non-occupationally exposed population. Toxicol Lett 137:65–83
93. Davison AG, Fayers PM, Taylor AJ, Venables KM, Darbyshire J, Pickering CA, Chettle DR, Franklin D, Guthrie CJ, Scott MC (1988) Cadmium fume inhalation and emphysema. Lancet 1:663–667
94. Mascagni P, Consonni D, Bregante G, Chiappino G, Toffoletto F (2003) Olfactory function in workers exposed to moderate airborne cadmium levels. Neurotoxicology 24:717–724
95. Åkesson A, Bjellerup P, Lundh T, Lidfeldt J, Nerbrand C, Samsioe G, Skerfving S, Vahter M (2006) Cadmium-induced effects on bone in a population-based study of women. Environ Health Perspect 114:830–834
96. Gallagher CM, Kovach JS, Meliker JR (2008) Urinary cadmium and osteoporosis in U.S. women $\geq$50 years of age: NHANES 1988–1994 and 1999–2004. Environ Health Perspect 116:1338–1343
97. Schutte R, Nawrot TS, Richart T, Thijs L, Vanderschueren D, Kuznetsova T, van Hecke E, Roels HA, Staessen JA (2008) Bone resorption and environmental exposure to cadmium in women: a population study. Environ Health Perspect 116:777–783
98. Järup L, Berglund M, Elinder CG, Nordberg G, Vahter M (1998) Health effects of cadmium exposure—a review of the literature and a risk estimate. Scan J Work Environ Health 24 (Suppl 1):1–51
99. Wittman R, Hu H (2002) Cadmium exposure and nephropathy in a 28-year old female metals worker. Environ Health Perspect 110:1261–1266
100. Becker K, Kaus S, Krause C, Lepom P, Schulz C, Seiwert M, Seifert B (2002) German Environmental Survey 1998 (GerES III): environmental pollutants in blood of the German population. Int J Hyg Environ Health 205:297–308

101. Mannino DM, Holguin F, Greves HM, Savage-Brown A, Stock AL, Jones RL (2004) Urinary cadmium levels predict lower lung function in current and former smokers: data from the third national health and nutrition examination survey. Thorax 59:194–198
102. Elinder CG, Järup L (1996) Cadmium exposure and health risks: recent findings. Ambio 25:370–373
103. Baselt RC, Cravey RH (1995) Disposition of toxic drugs and chemicals in man, 4th edn. Year Book Medical Publishers, Chicago, IL, pp 105–107
104. Baselt RC (2000) Disposition of toxic drugs and chemicals in man, 5th edn. Chemical Toxicology Institute, Foster City, CA
105. Singhal RL, Merali Z, Hrdina PD (1976) Aspects of the biochemical toxicology of cadmium. Fed Proc 35:75–80
106. Waalkes MP (1995) Cadmium and carcinogenesis. In: Berthan G (ed) Handbook on metal–-ligand interactions of biological fluids, vol 2. Dekker, New York, NY, pp 471–482
107. Waalkes MP, Misra RR (1996) Cadmium carcinogenicity and genotoxicity. In: Chang LW, Magos L, Suzuli T (eds) Toxicology of metals. CRC, Boca Raton, FL, pp 231–243
108. Waalkes MP, Rehm S (1992) Carcinogenicity of oral cadmium in the male Wistar (WF/NCr) rat: effect of chronic dietary zinc deficiency. Fundam Appl Toxicol 19:512–520
109. Stohs SJ, Bagchi D (1995) Oxidative mechanisms in the toxicity of metal ions. Free Radic Biol Med 18:321–336
110. Mitra RS (1984) Protein synthesis in *Escherichia coli* during recovery from exposure to low levels of Cd^{2+}. Appl Environ Microbiol 47:1012–1016
111. Blom A, Harder W, Matin A (1992) Unique and overlapping pollutant stress proteins of *Escherichia coli*. Appl Environ Microbiol 58:331–334
112. Ferianc P, Farewell S, Nyström T (1998) The cadmium-stress stimulon of *Escherichia coli* K-12. Microbiology 144:1045–1050
113. Coogan TP, Bare RM, Waalkes MP (1992) Cadmium-induced DNA strand damage in cultured liver cells: reduction in cadmium genotoxicity following zinc pretreatment. Toxicol Appl Pharmacol 113:227–233
114. Tsuzuki K, Sugiyama M, Haramaki N (1994) DNA single-strand breaks and cytotoxicity induced by chromate (VI), cadmium (II), and mercury (II) in hydrogen peroxide-resistant cell lines. Environ Health Perspect 102:341–342
115. Mukherjee S, Das SK, Kabiru W, Russell KR, Greaves K, Ademoyero AA, Rhaney F, Hills ER, Archibong AE (2002) Acute cadmium toxicity and male reproduction. Adv Reprod 6:143–155
116. Rossman TG, Roy NK, Lin WC (1992) Is cadmium genotoxic? IARC Sci Publ 118:367–375
117. Smith JB, Dwyer SC, Smith L (1989) Lowering extracellular pH evokes inositol polyphosphate formation and calcium mobilization. J Biol Chem 264:8723–8728
118. Th'evenod F, Jones SW (1992) Cadmium block of calcium current in frog sympathetic neurons. Biophys J 63:162–168
119. Suszkiw J, Toth G, Murawsky M, Cooper GP (1984) Effects of Pb^{2+} and Cd^{2+} on acetylcholine release and Ca^{2+} movements in synaptosomes and subcellular fractions from rat brain and Torpedo electric organ. Brain Res 323:31–46
120. Dally H, Hartwig A (1997) Induction and repair inhibition of oxidative DNA damage by nickel (II) and cadmium (II) in mammalian cells. Carcinogenesis 18:1021–1026
121. Abshire MK, Devor DE, Diwan BA, Shaughnessy JD Jr, Waalkes MP (1996) *In vitro* exposure to cadmium in rat L6 myoblasts can result in both enhancement and suppression of malignant progression *in vivo*. Carcinogenesis 17:1349–1356
122. Durnam DM, Palmiter RD (1981) Transcriptional regulation of the mouse metallothionein-I gene by heavy metals. J Biol Chem 256:5712–5716
123. Hwua Y, Yang J (1998) Effect of 3-aminotriazole on anchorage independence and mutagenicity in cadmium- and lead-treated diploid human fibroblasts. Carcinogenesis 19:881–888
124. Landolph J (1994) Molecular mechanisms of transformation of CH3/10T1/2C1 8 mouse embryo cells and diploid human fibroblasts by carcinogenic metal compounds. Environ Health Perspect 102:119–125

125. Nishijo M, Tawara K, Honda R, Nakagawa H, Tanebe K, Saito S (2004) Relationship between newborn size and mother's blood cadmium levels, Toyama, Japan. Arch Environ Health 59:22–25
126. Zhang YL, Zhao YC, Wang JX, Zhu HD, Liu QF, Fan YG, Wang NF, Zhao JH, Liu HS, Ou-Yang L, Liu AP, Fan TQ (2004) Effect of environmental exposure to cadmium on pregnancy outcome and fetal growth: a study on healthy pregnant women in China. J Environ Sci Health A Tox Hazard Subst Environ Eng 39:2507–2515
127. Jacobs JA, Testa SM (2005) Overview of chromium (VI) in the environment: background and history. In: Guertin J, Jacobs JA, Avakian CP (eds) Chromium (VI) handbook. CRC, Boca Raton, FL, pp 1–22
128. ATSDR (2008) Toxicological profile for chromium. U.S. Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry, Atlanta, GA
129. IARC (1990) Chromium, nickel and welding, vol 49, IARC monographs on the evaluation of carcinogenic risks to humans. IARC Scientific Publications, IARC, Lyon, France
130. U.S. EPA (1992) Integrated risk information system (IRIS). Environmental Criteria and Assessment Office, United States Environmental Protection Agency, Cincinnati, OH
131. Velma V, Vutukuru SS, Tchounwou PB (2009) Ecotoxicology of hexavalent chromium in freshwater fish: a critical review. Rev Environ Health 24:129–145
132. Cohen MD, Kargacin B, Klein CB, Costa M (1993) Mechanisms of chromium carcinogenicity and toxicity. Crit Rev Toxicol 23:255–281
133. Norseth T (1981) The carcinogenicity of chromium. Environ Health Perspect 40:121–130
134. Wang XF, Xing ML, Shen Y, Zhu X, Xu LH (2006) Oral administration of Cr(VI) induced oxidative stress, DNA damage and apoptotic cell death in mice. Toxicology 228:16–23
135. Guertin J (2005) Toxicity and health effects of chromium (all oxidation states). In: Guertin J, Jacobs JA, Avakian CP (eds) Chromium (VI) handbook. CRC, Boca Raton, FL, pp 216–234
136. OSHA (2006) Occupational exposure to hexavalent chromium. Occupational safety and health administration, Federal Register 71, Washington DC, pp 10099–10385
137. Singh J, Pritchard DE, Carlisle DL, Mclean JA, Montaser A, Orenstein JM, Patierno SR (1999) Internalization of carcinogenic lead chromate particles by cultured normal human lung epithelial cells: formation of intracellular lead-inclusion bodies and induction of apoptosis. Toxicol Appl Pharmacol 161:240–248
138. Langård S, Vigander T (1983) Occurrence of lung cancer in workers producing chromium pigments. Br J Ind Med 40:71–74
139. Costa M (1997) Toxicity and carcinogenicity of Cr(VI) in animal models and humans. Crit Rev Toxicol 27:431–442
140. Shelnutt SR, Goad P, Belsito DV (2007) Dermatological toxicity of hexavalent chromium. Crit Rev Toxicol 37:375–387
141. WHO/IPCS (1988) Environmental health criteria 61: chromium. World Health Organization, Geneva
142. Chen TL, Wise SS, Kraus S, Shaffiey F, Levine K, Thompson DW, Romano T, O'Hara T, Wise JP (2009) Particulate hexavalent chromium is cytotoxic and genotoxic to the North Atlantic right whale (*Eubalaena glacialis*) lung and skin fibroblasts. Environ Mol Mutagen 50:387–393
143. Connett PH, Wetterhahn KE (1983) Metabolism of carcinogenic chromate by cellular constituents. Struct Bond 54:93–24
144. De Flora S, Bagnasco M, Serra D, Zanacchi P (1990) Genotoxicity of chromium compounds: a review. Mutat Res 238:99–172
145. Dayan AD, Paine AJ (2001) Mechanisms of chromium toxicity, carcinogenicity and allergenicity: review of the literature from 1985 to 2000. Hum Exp Toxicol 20:439–451
146. De Mattia G, Bravi MC, Laurenti O, De Luca O, Palmeri A, Sabatucci A, Mendico G, Ghiselli A (2004) Impairment of cell and plasma redox state in subjects professionally exposed to chromium. Am J Ind Med 46:120–125

147. O'Brien TJ, Ceryak S, Patierno SR (2003) Complexities of chromium carcinogenesis: role of cellular response, repair and recovery mechanisms. Mutat Res 533:3–36
148. Kim E, Na KJ (1991) Nephrotoxicity of sodium dichromate depending on the route of administration. Arch Toxicol 65:537–541
149. Gumbleton M, Nicholls PJ (1988) Dose-response and time-response biochemical and histological study of potassium dichromate-induced nephrotoxicity in the rat. Food Chem Toxicol 26:37–44
150. Bagchi D, Hassoun EA, Bagchi M, Muldoon D, Stohs SJ (1995) Oxidative stress induced by chronic administration of sodium dichromate (Cr-VI) to rats. Comp Biochem Physiol 110C: 281–287
151. Bagchi D, Vuchetich PJ, Bagchi M, Hassoun EA, Tran MX, Tang L, Stohs SJ (1997) Induction of oxidative stress by chronic administration of sodium dichromate (chromium VI) and cadmium chloride (cadmium II) to rats. Free Radic Biol Med 22:471–478
152. Gambelunghe A, Piccinini R, Ambrogi M, Villarini M, Moretti M, Marchetti C, Abbritti G, Muzi G (2003) Primary DNA damage in chrome-plating workers. Toxicology 188:187–195
153. Goulart M, Batoreu MC, Rodrigues AS, Laires A, Rueff J (2005) Lipoperoxidation products and thiol antioxidants in chromium-exposed workers. Mutagenesis 20:311–315
154. Wise JP Sr, Wise SS, Little JE (2002) The cytotoxicity and genotoxicity of particulate and soluble hexavalent chromium in human lung cells. Mutat Res 517:221–229
155. Wise SS, Holmes AL, Ketterer ME, Hartsock WJ, Fomchenko E, Katsifis SP, Thompson WD, Wise JP Sr (2004) Chromium is the proximate clastogenic species for lead chromate-induced clastogenicity in human bronchial cells. Mutat Res 560:79–89
156. Xie H, Wise SS, Holmes AL, Xu B, Wakeman T, Pelsue SC, Singh NP, Wise JP Sr (2005) Carcinogenic lead chromate induces DNA double-strand breaks in human lung cells. Mutat Res 586:160–172
157. Zhitkovich A, Song Y, Quievryn G, Voitkun V (2001) Non-oxidative mechanisms are responsible for the induction of mutagenesis by reduction of Cr(VI) with cysteine: role of ternary DNA adducts in Cr(III)-dependent mutagenesis. Biochemistry 40:549–60
158. Katz SA, Salem H (1993) The toxicology of chromium with respect to its chemical speciation: a review. J Appl Toxicol 13:217–224
159. Patlolla AK, Armstrong N, Tchounwou PB (2008) Cytogenetic evaluation of potassium dichromate toxicity in bone marrow cells of Sprague-Dawley rats. Metal Ions Biol Med 10:353–358
160. Velma V, Tchounwou PB (2010) Chromium-induced biochemical, genotoxic and histopathologic effects in liver and kidney of goldfish, *Carassius auratus*. Mutat Res 698:43–51
161. Norseth T (1986) The carcinogenicity of chromium and its salts. Br J Ind Med 3:649–651
162. Gabby PN (2006) Lead. In: Mineral commodity summaries, U.S. Geological Survey. Reston, VA. Online available at http://minerals.usgs.gov/minerals/pubs/commodity /lead/lead_mcs05.pdf
163. Gabby PN (2003) "Lead." Environmental defense, "Alternatives to Lead-Acid Starter Batteries," pollution prevention fact sheet. Online available at http://www.cleancarcampaign.org/FactSheet_BatteryAlts.pdf
164. CDC (1991) Preventing lead poisoning in young children: a statement by the centers for disease control. Centers for Disease Control and Prevention, Atlanta, GA
165. Jacobs DE, Clickner RP, Zhou JY, Viet SM, Marker DA, Rogers JW, Zeldin DC, Broene P, Friedman W (2002) The prevalence of lead-based paint hazards in U.S. housing. Environ Health Perspect 110:A599–A606
166. Farfel MR, Chisolm JJ Jr (1991) An evaluation of experimental practices for abatement of residential lead-based paint: report on a pilot project. Environ Res 55:199–212
167. CDC (2001) Managing elevated blood lead levels among young children: recommendations from the advisory committee on childhood lead poisoning prevention. Centers for Disease Control and Prevention, Atlanta, GA
168. Lanphear BP, Matte TD, Rogers J, Clickner RP, Dietz B, Bornschein RL, Succop P, Mahaffey KR, Dixon S, Galke W, Rabinowitz M, Farfel M, Rohde C, Schwartz J, Ashley P, Jacobs DE (1998) The contribution of lead-contaminated house dust and

residential soil to children's blood lead levels. A pooled analysis of 12 epidemiologic studies. Environ Res 79:51–68
169. Charney E, Sayre J, Coulter M (1980) Increased lead absorption in inner city children: where does the lead come from? Pediatrics 6:226–231
170. ATSDR (1999) Toxicological profile for Lead. Public Health Service, U.S. Department of Health and Human Services, Agency for Toxic Substances and Disease Registry, Atlanta, GA
171. ATSDR (1992) Case studies in environmental medicine—Lead toxicity. Public Health Service, U.S. Department of Health and Human Services, Agency for Toxic Substances and Disease Registry, Atlanta, GA
172. Flora SJS, Flora GJS, Saxena G (2006) Environmental occurrence, health effects and management of lead poisoning. In: Cascas SB, Sordo J (eds) Lead: chemistry, analytical aspects, environmental impacts and health effects. Elsevier, The Netherlands, pp 158–228
173. Pirkle JL, Brady DJ, Gunter EW, Kramer RA, Paschal DC, Flegal KM, Matte TD (1994) The decline in blood lead levels in the United States: The National Health and Nutrition Examination Surveys (NHANES). J Am Med Assoc 272:284–291
174. Pirkle JL, Kaufmann RB, Brody DJ, Hickman T, Gunter EW, Paschal DC (1998) Exposure of the U.S. population to lead: 1991–1994. Environ Health Perspect 106:745–750
175. U.S. EPA (2002) Lead compounds. Technology transfer network—air toxics website. Online available at http://www.epa.gov/cgi-bin/epaprintonly.cgi
176. Kaul B, Sandhu RS, Depratt C, Reyes F (1999) Follow-up screening of lead-poisoned children near an auto battery recycling plant, Haina, Dominican Republic. Environ Health Perspect 107:917–920
177. Ong CN, Phoon WO, Law HY, Tye CY, Lim HH (1985) Concentrations of lead in maternal blood, cord blood, and breast milk. Arch Dis Child 60:756–759
178. Corpas I, Gaspar I, Martinez S, Codesal J, Candelas S, Antonio MT (1995) Testicular alterations in rats due to gestational and early lactational administration of lead. Report Toxicol 9:307–313
179. Andrews KW, Savitz DA, Hertz-Picciotto I (1994) Prenatal lead exposure in relation to gestational age and birth weight: a review of epidemiologic studies. Am J Ind Med 26:13–32
180. Huel G, Tubert P, Frery N, Moreau T, Dreyfus J (1992) Joint effect of gestational age and maternal lead exposure on psychomotor development of the child at six years. Neurotoxicology 13:249–254
181. Litvak P, Slavkovich V, Liu X, Popovac D, Preteni E, Capuni-Paracka S, Hadzialjevic S, Lekic V, Lolacono N, Kline J, Graziano J (1998) Hyperproduction of erythropoietin in nonanemic lead-exposed children. Environ Health Perspect 106:361–364
182. Amodio-Cocchieri R, Arnese A, Prospero E, Roncioni A, Barulfo L, Ulluci R, Romano V (1996) Lead in human blood form children living in Campania, Italy. J Toxicol Environ Health 47:311–320
183. Hertz-Picciotto I (2000) The evidence that lead increases the risk for spontaneous abortion. Am J Ind Med 38:300–309
184. Apostoli P, Kiss P, Stefano P, Bonde JP, Vanhoorne M (1998) Male reproduction toxicity of lead in animals and humans. Occup Environ Med 55:364–374
185. Flora SJS, Saxena G, Gautam P, Kaur P, Gill KD (2007) Lead induced oxidative stress and alterations in biogenic amines in different rat brain regions and their response to combined administration of DMSA and MiADMSA. Chem Biol Interact 170:209–220
186. Hermes-Lima M, Pereira B, Bechara EJ (1991) Are free radicals involved in lead poisoning? Xenobiotica 8:1085–1090
187. Jiun YS, Hsien LT (1994) Lipid peroxidation in workers exposed to lead. Arch Environ Health 49:256–259
188. Bechara EJ, Medeiros MH, Monteiro HP, Hermes-Lima M, Pereira B, Demasi M (1993) A free radical hypothesis of lead poisoning and inborn porphyrias associated with 5-aminolevulinic acid overload. Quim Nova 16:385–392

189. Yedjou CG, Steverson M, Paul Tchounwou PB (2006) Lead nitrate-induced oxidative stress in human liver carcinoma ($HepG_2$) cells. Metal Ions Biol Med 9:293–297
190. Yedjou CG, Milner J, Howard C, Tchounwou PB (2010) Basic apoptotic mechanisms of lead toxicity in human leukemia (HL-60) cells. Int J Environ Res Public Health 7:2008–2017
191. Goldstein G (1993) Evidence that lead acts as a calcium substitute in second messenger metabolism. Neurotoxicology 14:97–102
192. Simons T (1993) Lead–calcium interactions in cellular lead toxicity. Neurotoxicology 14: 77–86
193. Vijverberg HPM, Oortgiesen M, Leinders T, van Kleef RGDM (1994) Metal interactions with voltage- and receptor-activated ion channels. Environ Health Perspect 102:153–158
194. Schanne FA, Long GJ, Rosen JF (1997) Lead induced rise in intracellular free calcium is mediated through activation of protein kinase C in osteoblastic bone cells. Biochim Biophys Acta 1360:247–254
195. Waalkes MP, Hiwan BA, Ward JM, Devor DE, Goyer RA (1995) Renal tubular tumors and atypical hyperplasias in B6C3F, mice exposed to lead acetate during gestation and lactation occur with minimal chronic nephropathy. Cancer Res 55:5265–5271
196. Goyer RA (1993) Lead toxicity: current concerns. Environ Health Prospect 100:177–187
197. IARC (1987) Overall evaluation of carcinogenicity: an updating of monographs. IARC monographs on the evaluation of carcinogenic risks to humans, vols 1–42, Suppl 7. International Agency for Research on Cancer, Lyon
198. Yang JL, Wang LC, Chamg CY, Liu TY (1999) Singlet oxygen is the major species participating in the induction of DNA strand breakage and 8-hydroxy-deoxyguanosine adduct by lead acetate. Environ Mol Mutagen 33:194–201
199. Lin RH, Lee CH, Chen WK, Lin-Shiau SY (1994) Studies on cytotoxic and genotoxic effects of cadmium nitrate and lead nitrate in Chinese hamster ovary cells. Environ Mol Mutagen 23:143–149
200. Dipaolo JA, Nelson Rh, Casto BC (1978) *In-vitro* neoplastic transformation of Syrian hamster cell by lead acetate and its relevance to environmental carcinogenesis. Br J Cancer 38:452–455
201. Roy N, Rossman T (1992) Mutagenesis and comutagenesis by lead compounds. Mutat Res 298:97–103
202. Wise JP, Orenstein JM, Patierno SR (1993) Inhibition of lead chromate clastogenesis by ascorbate: relationship to particle dissolution and uptake. Carcinogenesis 14:429–434
203. Clarkson TW, Magos L, Myers GJ (2003) The toxicology of mercury-current exposures and clinical manifestations. N Engl J Med 349:1731–1737
204. Guzzi G, LaPorta CAM (2008) Molecular mechanisms triggered by mercury. Toxicology 244: 1–12
205. Dopp E, Hartmann LM, Florea AM, Rettenmier AW, Hirner AV (2004) Environmental distribution, analysis, and toxicity of organometal (loid) compounds. Crit Rev Toxicol 34: 301–333
206. Sarkar BA (2005) Mercury in the environment: effects on health and reproduction. Rev Environ Health 20:39–56
207. Zahir A, Rizwi SJ, Haq SK, Khan RH (2005) Low dose mercury toxicity and human health. Environ Toxicol Pharmacol 20:351–360
208. Holmes P, Hames KAF, Levy LS (2009) Is low-level mercury exposure of concern to human health? Sci Total Environ 408:171–182
209. Tchounwou PB, Ayensu WK, Ninashvilli N, Sutton D (2003) Environmental exposures to mercury and its toxicopathologic implications for public health. Environ Toxicol 18: 149–175
210. U.S. EPA (1997) Mercury study report to congress. Online available at http://www.epa.gov/mercury/report.htm
211. Sanfeliu C, Sebastia J, Cristofol R, Rodriquez-Farre E (2003) Neurotoxicity of organomercurial compounds. Neurotox Res 5:283–305

212. Valko M, Morris H, Cronin MTD (2005) Metals, toxicity, and oxidative stress. Curr Med Chem 12:1161–1208
213. Valko M, Rhodes CJ, Monocol J, Izakovic-Mazur M (2006) Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 160:1–40
214. Shenker BJ, Guo TL, Shapiro IM (2000) Mercury-induced apoptosis in human lymphoid cells: evidence that the apoptotic pathway is mercurial species dependent. Environ Res 84:89–99
215. Palmeira CM, Madeira VMC (1997) Mercuric chloride toxicity in rat liver mitochondria and isolated hepatocytes. Environ Toxicol Pharmacol 3:229–235
216. Lund BO, Miller DM, Woods JS (1991) Mercury induced H_2O_2 formation and lipid peroxidation *in vitro* in rat kidney mitochondria. Biochem Pharmacol 42:S181–S187
217. Clarkson TW, Magos L (2006) The toxicology of mercury and its chemical compounds. Crit Rev Toxicol 36:609–662
218. Sunja Kim S, Dayani L, Rosenberg PA, Li J (2010) RIP1 kinase mediates arachidonic acid-induced oxidative death of oligodendrocyte precursors. Int Physiol Pathophysiol Pharmacol 2:137–147
219. Lash LH, Putt DA, Hueni SE, Payton SG, Zwicki J (2007) Interactive toxicity of inorganic mercury and trichloroethylene in rat and human proximal tubules (Effects of apoptosis, necrosis, and glutathione status). Toxicol Appl Pharmacol 221:349–362
220. Rooney JPK (2007) The role of thiols, dithiols, nutritional factors and interacting ligands in the toxicology of mercury. Toxicology 234:145–156
221. Agarwal R, Goel SK, Chandra R, Behari JR (2010) Role of viamin E in preventing acute mercury toxicity in rat. Environ Toxicol Pharmacol 29:70–78
222. Leaner VD, Donninger H, Birrer MJ (2007) Transcription factors as targets for cancer therapy: AP-1 a potential therapeutic target. Curr Cancer Therap Rev 3:1–6
223. Marnett LJ (2000) Oxyradicals and DNA damage. Carcinogenesis 21:361–370
224. Zalups RK, Koropatnik J (2000) Molecular biology and toxicology of metals. Taylor & Francis, London
225. Magos L, Clarkson TW (2006) Overview of the clinical toxicity of mercury. Ann Clin Biochem 43:257–268
226. Valko M, Izakovic M, Mazur M, Rhodes CJ, Tesler J (2004) Role of oxygen radicals in DNA damage and cancer incidence. Mol Cell Biochem 266:79–110
227. Crespo-Lopez MR, Macedo GL, Pereira SID, Arrifano GPF, Picano-Dinc DLW, doNascimento JLM, Herculano AM (2009) Mercury and human genotoxicity: critical considerations and possible molecular mechanisms. Pharmacol Res 60:212–220
228. Ogura H, Takeuchi T, Morimoto KA (1996) A comparison of the 8-hydroxyl-deoxyguanosine, chromosome aberrations and micronucleus techniques for the assessment of the genotoxicity of mercury compounds in human blood lymphocytes. Mutat Res 340:175–182
229. Inoue M, Sato EF, Nishikawa M, Park AM, Kari Y, Imada I, Utsumi K (2003) Mitochondrial generation of reactive oxygen species and its role in aerobic life. Curr Med Chem 10: 2495–2505
230. Pinheiro MCN, Macchi BM, Vieira JLF, Oikawa T, Amoras WW, Santos EO (2008) Mercury exposure and antioxidant defenses in women: a comparative study in the Amazon. Environ Res 107:53–59
231. Amorim MI, Mergler D, Bahia MO, Miranda H, Lebel J (2000) Cytogenetic damage related to low levels of methylmercury contamination in the Brazilian Amazon. Ann Acad Bras Cienc 72:497–507
232. Rana SVS (2008) Metals and apoptosis: recent developments. J Trace Elem Med Biol 22: 262–284
233. López Alonso M, Prieto Montaña F, Miranda M, Castillo C, Hernández J, Luis Benedito J (2004) Interactions between toxic (As, Cd, Hg and Pb) and nutritional essential (Ca, Co, Cr, Cu, Fe, Mn, Mo, Ni, Se, Zn) elements in the tissues of cattle from NW Spain. Biometals 17:389–397

234. Abdulla M, Chmielnicka J (1990) New aspects on the distribution and metabolism of essential trace elements after dietary exposure to toxic metals. Biol Trace Elem Res 23: 25–53
235. Wang G, Fowler BA (2008) Roles of biomarkers in evaluating interactions among mixtures of lead, cadmium and arsenic. Toxicol Appl Pharmacol 233:92–99
236. Nordberg GF, Jin T, Hong F, Zhang A, Buchet JP, Bernard A (2005) Biomarkers of cadmium and arsenic interactions. Toxicol Appl Pharmacol 206:191–197

Toxicology of Ambient Particulate Matter

Damiën van Berlo, Maja Hullmann, and Roel P.F. Schins

Abstract It is becoming increasingly clear that inhalation exposure to particulate matter (PM) can lead to or exacerbate various diseases, which are not limited to the lung but extend to the cardiovascular system and possibly other organs and tissues. Epidemiological studies have provided strong evidence for associations with chronic obstructive pulmonary disease (COPD), asthma, bronchitis and cardiovascular disease, while the evidence for a link with lung cancer is less strong. Novel research has provided first hints that exposure to PM might lead to diabetes and central nervous system (CNS) pathology. In the current review, an overview is presented of the toxicological basis for adverse health effects that have been linked to PM inhalation. Oxidative stress and inflammation are discussed as central processes driving adverse effects; in addition, profibrotic and allergic processes are implicated in PM-related diseases. Effects of PM on key cell types considered as regulators of inflammatory, fibrotic and allergic mechanisms are described.

Keywords Diesel exhaust particles · Inflammation · NF-κB · Organics · Oxidative stress · Particulate matter · $PM_{2.5}$ · PM_{10} · Transition metals

D. van Berlo, M. Hullmann, and R.P.F. Schins (✉)
Particle Research, Institut für Umweltmedizinische Forschung (IUF), Heinrich-Heine University Düsseldorf, Auf'm Hennekamp 50, 40225 Düsseldorf, Germany
e-mail: roel.schins@uni-duesseldorf.de

A. Luch (ed.), *Molecular, Clinical and Environmental Toxicology*,
Experientia Supplementum 101, DOI 10.1007/978-3-7643-8340-4_7,

Particulate Matter: History and Characterisation

Why Particulates Matter: Historical Background

Since the earliest stages of humanity, exposure to aerosols (liquid or solid particulates suspended in a gas) derived from natural sources such as wildfires, dust storms, sea spray and volcanic activity has occurred. When humans learned to use fire in a controlled fashion approximately 400,000 years ago [1], exposure to combustion-derived particles increased due to the application of fire for cave warming and indoor cooking. Much later in human history, a major boost in the concentration of ambient particulate matter (PM) was caused by industrialisation and by the invention and popularisation of the internal combustion engine, commonly used to power the automobile. As of today, due to the ubiquitous presence of particles in outdoor air, this heterogeneous exposure is all but inevitable.

The understanding of ambient air pollution, which contains a mixture of potentially harmful components including nitrogen oxides, sulphur dioxide as well as particulate material, as a hazard to human health has increased substantially due to several acute episodes of severe smog. For instance, from the 1st to the 5th of December 1930, specific atmospheric conditions coupled to industrial generation of pollutants from steel mills, coke ovens, foundries and smelters caused the accumulation of high ambient air pollution levels in the Meuse Valley [2]. Mortality rates quickly rose tenfold, with 60 lives being lost [3]. A more widespread and more famous smog episode took place in Greater London, from the 5th to the 9th of December 1952. Government reports spoke of 3,000 extra deaths on top of the normal mortality rates during the first 3 weeks of this notorious December, a threefold augmented death rate [4]. The total amount of deaths due to "the Big Smoke" could be as high as 12,000 because mortality rates were increased until well into 1953 [5]. The principal culprits for the adverse health effects are considered to be sulphur dioxide and particulate matter. Severe acute episodes of strongly increased air pollution, leading to markedly increased morbidity and mortality, are not just a thing of the past. In the winters of 1991, 1994 and 1997, London residents have experienced significant air pollution episodes. From the 12th to the 15th of December 1991, London was struck by a dense smog incorporating high levels of nitrogen dioxide and PM. The PM concentration reached levels of up to 228 $\mu g/m^3$; mortality was amplified by approximately 10% [6].

The significance of the health hazard posed by ambient particulate matter was demonstrated when Pope *et al.* observed decreased hospital admissions in Utah Valley after the closing of a steel mill, which produced copious amounts of metal-rich particles and represented the largest single particle pollution source in the valley [7]. Correlations were robust and especially strong for children; their admissions were two to three times higher during the winters when the mill was open, compared to the winter when the mill was closed because of a labour dispute [7]. Toxicological support for these epidemiological observations was provided by investigations with aqueous extracts of the Utah Valley PM samples that were

used to treat cell cultures and experimental animals, as well as to expose human volunteers. Exemplary, the metal-rich PM, sampled in the years when the steel mill was operating, had a stronger ability to induce the pro-inflammatory interleukins IL-6 and IL-8 from human bronchial epithelial cells (BEAS-2B), while humans exposed to aqueous extracts from these PM samples developed an inflammatory response in the lower respiratory tract [8]. Following on the publication of these landmark Utah Valley studies, a large body of scientific evidence has been generated supporting the findings on particle-induced adverse health effects. Epidemiological studies have delivered convincing evidence for a positive association between exposure to particulate matter and respiratory, cardiovascular and malignant lung disease and mortality [9–11]. For instance, after a 1990 ban on coal sales in Dublin, adjusted non-trauma death rates decreased by 5.7%, respiratory deaths by 15.5% and cardiovascular deaths by 10.3%. The average black smoke concentration in Dublin was reduced by as much as 70% [12].

What Is Particulate Matter?

Particulate air pollution comprises a heterogeneous mixture of substances including carbon, metals, nitrates, sulphates and particulate organic matter; its composition varies according to the predominant source of particles, season and prevailing weather conditions [13, 14]. In present-day Western urban environments, a major contributor to PM is engine exhaust released by diesel and petrol vehicles. Until several decades ago, PM concentrations in ambient air were typically measured as total suspended particles (TSP) [15]. In 1987, the EPA revised the National Ambient Air Quality Standard (NAAQS) for PM, forwarding PM_{10} as a superior index, since this fraction is more representative of the particles that can actually be inhaled and enter the human lung [15]. PM_{10} can be defined as particulate matter with a mean aerodynamic diameter of 10 μm. The aerodynamic diameter is defined as the diameter of a spherical particle having a density of 1 g/cm^3 that has the same inertial properties in the gas as the particle of interest. PM_{10} is a complex mixture originating from both natural and anthropogenic sources. It consists of a broad range of particulate components as well as a plethora of substances bound to the core of the particles [14]. PM_{10} can be split up further in several size fractions, based on the aerodynamic diameter resulting from collection using specific particle matter samplers: coarse (2.5–10 μm), fine (0.1–2.5 μm) and ultrafine (<0.1 μm). The latter size fraction is also referred to by toxicologists as ultrafine particles (UFP). Accordingly, in the recently emerging field of nanotoxicology, a distinction can be made between ambient and engineered nanoparticles (see chapter Nanoparticles: A Challenge for Toxicological Risk Assessment? authored by Haase *et al.*). An alternative approach to classify particles is according to a modal characterisation, e.g. coarse, accumulation, Aitken and nucleation modes [16]. These roughly correspond to particle fractions with a diameter of 1–50 μm, 0.1–1 μm, 10–100 nm and <10 nm, respectively. Both characterisations are shown in Fig. 1.

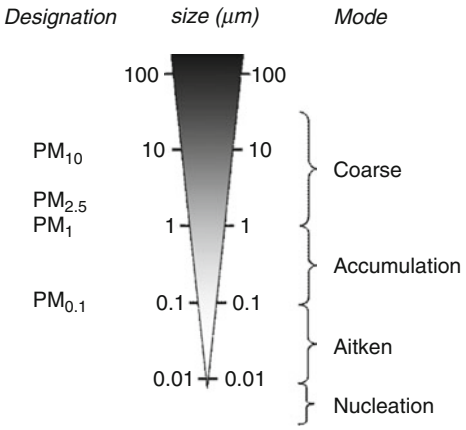


Fig. 1 Characterisation and subdivision of particulate matter

PM varies widely in composition and physicochemical properties. Coarse particles are often derived from natural sources and contain mostly crustal elements, while the fine fraction of UFP is typically dominated by particles from anthropogenic sources including combustion processes and hence may contain large amounts of organic carbon compounds such as polycyclic aromatic hydrocarbons (PAHs) and quinones [17]. For the investigation of possible mechanisms of PM-induced adverse health effects, model particles have been used in many toxicological studies, which represent different components of the complicated multiple entity that is PM. Commercially available particulate materials such as carbon black and titanium dioxide represent a model for the carbon core of combustion-derived particles and poorly soluble (nano)particles in general. Residual oil fly ash (ROFA), a by-product of power plant and industry fuel–oil combustion that is very rich in metals, has been used in several studies to evaluate the role of transition metals in the toxicity of PM. Besides such types of model particles, real-life particulates from ambient air, or their organic or aqueous extracts, have been used for toxicological evaluation. Such PM can be collected using (high volume) particle samplers for subsequent evaluation in cell culture studies, animal experiments or human volunteer studies. An alternative approach which has been developed to evaluate on-site effects has been to concentrate particles before inhalation exposure studies in laboratory animals or human volunteers: these have been referred to as concentrated ambient particles (CAP). An obvious disadvantage of the use of real-life PM in terms of reproducibility in mechanistic studies is that its composition and hence its toxic potency may strongly depend on weather and anthropogenic conditions (e.g. wind direction, season, traffic density). In fact, however, this variability has been the basis of specific toxicological investigations, such as in the Utah Valley studies [8]. Diesel exhaust particles (DEP) form a special group among the various types of particles that are used to address mechanisms of toxicity of PM. They contain considerable amounts of metals and organics onto their carbonaceous core and can be generated and subsequently sampled in a highly

Table 1 Particle types used to investigate particulate matter (PM) properties relevant for human health

Particle type	Category	Representative for	Tested in	References
Concentrated ambient particles (CAP)	Real life (direct/on-site exposure)		Human volunteers, rats, mice, cell culture	[128, 297–300]
PM sampled on filters or in suspensions ("biosamples")	Real life (tested after storage and/or extraction)		Human volunteers, rats, mice, cell culture	[33, 145, 245, 301, 302]
Diesel engine exhaust particles (DEP)	Model	UFP, rich in metals and PAHs	Rats, mice, cell culture	[90, 303, 304]
Residual oil fly ash (ROFA), coal fly ash	Model	Metal-rich particles (relatively soluble)	Rats, mice, cell culture	[305–308]
Carbon black (fine, ultrafine)	Model	Fine PM, UFP (combustion-derived nanoparticles)	Rats, mice, cell culture	[309–312]
Titanium dioxide (fine, ultrafine)	Model	Poorly soluble particles (fine *versus* ultrafine)	Rats, mice, cell culture	[313–315]

controlled and reproducible manner for toxicology studies. Accordingly, while representing a dominant component of PM in urban environments, DEP are typically used as model particles to represent UFP. An overview of the most common experimental approaches used to address PM toxicity is provided in Table 1.

Besides pure particle exposures, a lot of studies have been performed investigating the contribution of diesel engine exhaust to the adverse health effects that are attributed to air pollution. Of course, this is not a "pure" particle exposure, incorporating a large amount of non-particulate compounds such as carbon monoxide (CO), nitric oxides (NO, NO_2), sulphur dioxide (SO_2), hydrocarbons, formaldehyde and transition metals [18]. There may be interactions between solid and gaseous constituents, and gaseous components can form particulates. Also, synergistic effects can be elicited, like those reported for PM-bound metals and organics [19]; both are present in varying amounts in diesel engine exhaust as particulates and gases. Therefore, these findings could be of relevance for adverse health effects resulting from PM exposure. An advantage of diesel engine exhaust exposure is that studies feature inhalation, which results in a relatively homogeneous distribution of inhaled particles and is of course closer to the real-life situation than a bolus application of DEP into the lung as can be achieved by pharyngeal aspiration or intratracheal instillation in, for instance, rodents.

Inhalation of Particulate Matter

In European cities, PM_{10} levels nowadays typically vary between 15 and 60 μg/m^3 [20]. The adult tidal volume is 0.4–0.5 L for women and men, respectively, which means that every breath of air during normal activity contains 6–30 ng PM_{10}. The average human breathes once every 5 s, so 0.1–0.5 mg of particles passes the respiratory tract every day. Of course, only a fraction of this amount actually deposits in the lung [21]. In non-Western countries, particle concentrations can be much higher. In the centre of Beijing, the monthly average concentration of the $PM_{2.5}$ fraction alone (roughly 50–80% of PM_{10} mass) often reaches levels of over 100 μg/m^3, while during air pollution episodes, they can be as high as 300 μg/m^3 [22].

Particle size largely determines penetration depth into the airways, i.e. which region can be affected [21]. PM_{10} is also known as the thoracic or inhalable fraction because particles with an aerodynamic diameter smaller than 10 μm can be inhaled and are thus capable of bypassing the human upper respiratory tract and enter the lung. Larger particles deposit mainly in the nasal region and are rapidly cleared. $PM_{2.5}$ is often referred to as fine particles, or the respirable fraction of PM. These smaller particles are capable of reaching the most distal regions of the lung, depositing in the bronchiolar and alveolar regions. $PM_{0.1}$ (or UFP) can of course also enter the alveoli although extremely small particles behave like a gas and can therefore be easily exhaled, limiting their deposition compared to particles of greater aerodynamic size. The largest pulmonary deposition of UFP has been reported for particles with a size of 20 nm, with 50% depositing in the alveolar region. However, a relatively large UFP percentage can be deposited in the upper airways including the nasal region [16], depending on the particle size.

Particulate Matter: Adverse Health Effects

Acute and Long-Term Effects

Regarding the effects of human exposure to PM, both long-term and acute effects need to be assessed. Periods with pronounced acute particulate air pollution due to specific climatologic circumstances, such as the severe pollution episode in London, are known to lead to augmented mortality rates and increased incidence of cardiopulmonary diseases. Aside from such acute events, people are chronically exposed to lower concentrations of particulate air pollution in ambient air. Importantly, high acute exposures to particulate matter also contribute to chronic adverse health effects [23]. Reflecting this dual character of PM-associated adverse health effects, separate short-term and long-term guidelines have been provided by the WHO relating to the PM concentrations in ambient air.

Table 2 Particulate matter (PM) characteristics that are mainly implicated in adverse health effects

Constituent	Mechanism	References
Metals	ROS formation via Fenton and Haber–Weiss chemistry	[28–30]
Organics (PAHs, nitro-PAHs, quinones)	DNA adducts, quinone cycling (ROS formation)	[34, 35]
Size and surface area	ROS formation on large reactive surface	[31]
Endotoxin	Activation of immune cells via toll-like receptors	[13, 33]

There is plentiful epidemiological evidence for associations of PM inhalation with (exacerbation of) asthma, bronchitis, chronic obstructive pulmonary disease (COPD) and cardiovascular disease. Lung cancer has also been linked to PM exposure, although associations are less robust. Novel results suggest that diabetes can be added to this list of diseases. A first hint was published in 2002. PM_{10} exposure was identified as factor that could potentially contribute to the development of type 1 diabetes in young children (<5 years of age) [24]. In two very recent studies, associations have been found between particulate air pollution and the incidence or prevalence of diabetes. One study reported an association between diabetes prevalence and $PM_{2.5}$ levels. A 10 $\mu g/m^3$ increase in $PM_{2.5}$ concentration corresponds to a 1% prevalence increase [25]. Another study found an association between the incidence of type 2 diabetes in elderly women and traffic-related particulate air pollution [26].

The estimated number of worldwide deaths that has been attributed to PM exposure on a yearly basis was over 500,000 in 1994 [27]. Factors/characteristics of PM that have been held responsible for the observed adverse effects include transition metal content (e.g. Fe, Ni, Cu, Co, Cr) and iron mobilisation [28–30], particle size and surface area [31, 32], endotoxin contamination [13, 33] and organic compounds such as PAHs [34, 35]. These are summarised in Table 2. UFP are hypothesised to be the most dangerous PM fraction. They possess the largest surface area per unit of mass and the highest hydrocarbon content compared to the other fractions. UFP that are generated through engine fuel combustion consist of a carbon core to which a variety of metals and organic chemicals are adsorbed, including known mutagens and carcinogens [36]. However, the contribution of larger particles to PM-associated pathology should not be entirely disregarded.

Susceptibility

Of particular relevance is the fact that especially susceptible individuals suffer from PM exposure. This susceptibility depends on age (e.g. [37]), health status (e.g. [38]) and genetic makeup (e.g. [39]). Most of the mortalities during acute air pollution

episodes are observed among the elderly, the very young and people with pre-existing (cardiopulmonary) disease. In addition to these vulnerable populations, attempts have been made to identify genetic polymorphisms that might render carriers susceptible to the adverse health effects of PM. The major findings in this regard are summarised in Table 3. This diverse list of polymorphisms is still somewhat limited, and more research is needed to shed more light on the susceptibility matter at the molecular level.

Pulmonary Effects

Many studies have provided experimental support for the associations found in epidemiological studies between PM exposure and adverse pulmonary effects. Upon deposition, PM can trigger inflammatory processes, potentially leading to airway obstruction, compromised gas exchange and the exacerbation of pre-existing conditions such as asthma or chronic bronchitis [10, 40, 41]. PM exposure is associated with lung pathology including COPD, bronchitis, asthma and lung cancer.

Defining characteristics of COPD are airflow obstruction and exacerbation. Exacerbation, a sudden deterioration of the patient's condition which often leads to hospitalisation, has been associated with PM_{10} levels [42–44]. Comparison of lung tissues obtained from Mexico City residents to those from Vancouver citizens, areas featuring high and low PM_{10} levels, respectively, resulted in the detection of considerable small airway fibrosis in the first group which was all but absent in the second [45]. Profibrotic mechanisms are involved in bronchitis, COPD and asthma [46]. The hallmark of fibrosis is disturbed wound healing, leading to excessive deposition of collagen by fibroblasts. During normal wound healing, inflammatory cells, which eliminate tissue debris, necrotic/apoptotic cells and invading micro-organisms, produce cytokines and chemokines that are mitogenic and chemotactic for endothelial and epithelial cells. Endothelial and epithelial cells then migrate towards the centre of the lesion. Meanwhile, pulmonary inflammatory cells release growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β), which are strong fibroblast stimulators [47, 48]. In mice, PDGF was shown to be essentially involved in airway thickening elicited by DEP [49]. Probably, the most important cytokine involved in fibrosis is TGF-β [50]. Once activated, fibroblasts transform into myofibroblasts that promote wound contraction. Subsequently, epithelial/endothelial cells divide, and thus, the damaged tissue is regenerated [51]. However, in the case of chronic pulmonary inflammation, persistent tissue damage occurs, leading to augmented proliferation of fibroblasts and an exaggerated deposition of extracellular matrix (ECM) components, especially collagen, the accumulation of which is the hallmark of fibrosis [52]. Due to an imbalance in collagen synthesis *versus* degradation with the scale tipping towards the first, the flexibility of the lung becomes progressively limited, a process that is severely debilitating and can finally result in mortality.

Table 3 Genetic polymorphisms modifying the risk of adverse PM-associated health effects

Gene	Function	Effect of polymorphism	References
Glutathione *S*-transferase (GST), heme oxygenase-1 (HMOX1) genes	Antioxidant genes	Modification of attenuation of lung function declines after PM_{10} reduction	[316]
Apolipoprotein E (APOE), lipoprotein lipase (LPL), vascular endothelial growth factor (VEGF)	Lipid metabolism, growth factor	Stronger particle effects on heart rate variability observed in carriers of wild-type APOE, LPL and VEGF genes than in those with hetero- or homozygous types	[317]
PHD finger protein 11 (PHF11), matrix metalloprotease 1 (MMP1), inositol 1,4,5-triphosphate receptor, type 2 (ITPR2)	T cell regulation, extracellular matrix breakdown, calcium channel/receptor	$PM_{2.5}$-associated changes in systolic and diastolic blood pressure are modified	[318]
p53, cyclin D1 (CCND1)	Tumour suppressor gene, cell cycle control gene	Modification of attenuation of age-related lung function declines after PM_{10} reduction	[319]
Catalase, myeloperoxidase (MPO)	Antioxidant gene; innate immune response gene	Risk of respiratory-related school absences elevated for children with the CAT (G/G) and MPO (G/A or A/A) genes	[320]
Fibrinogen	Marker for systemic inflammation	Subjects with variants of fibrinogen genes may have increased risks due to constitutionally higher fibrinogen concentrations and augmented response to ambient particulate matter	[321]
C677T methylenetetrahydrofolate reductase (MTHFR) and C1420T cytoplasmic serine hydroxymethyl-transferase (cSHMT)	Metabolic genes	Modification of $PM_{2.5}$-induced changes in heart rate variability	[322]
Hemochromatosis (HFE)	Modulates lung uptake of iron/divalent cations, reducing their toxicity	Effect of $PM_{2.5}$ on heart rate variability was shielded in subjects with at least 1 copy of an HFE variant compared with wild-type subjects	[323]

The profibrotic potential of ambient particles depends on size and composition. The presence of transition metals, endotoxins and PAHs has been linked to the PM-induced activation of profibrotic pathways [46].

Acute inflammatory responses induced by a quick rise in $PM_{2.5}$ levels can provoke asthma flares, characterised by higher symptom scores and increased need for medication and hospitalisation [10, 41, 53]. Also, long-term PM exposure might lead to increased asthma prevalence. PM can act as an adjuvant for allergic sensitisation to environmental allergens, e.g. pollen [40, 54, 55]. In mice, co-exposure to ovalbumin and carbon black particles augments presence of myeloid dendritic cells and expression of CD80 and CD86 in the peribronchial lymph nodes [56]. In lungs of mice co-exposed to DEP and ovalbumin, enhanced expression of MHC class II, CD11c, DEC205 (dendritic cell markers), CD80 and CD86 (co-stimulatory molecules) was found, indicating maturation and activation of dendritic cells [57]. In another study, DEP exposure induced dendritic cell maturation and migration to the lung. Also, enhanced migration to the mediastinal lymph node (MLN) was observed, which led to an induction of T-cell recruitment in the MLN [58].

Exposure to particulate matter [11, 59] and diesel engine exhaust particles [60] has been associated with the increased occurrence of pulmonary neoplasms. Physicochemical particle properties that can contribute to genotoxicity include particle size, solubility [61] as well as surface-bound carcinogens [36]. In the pathologic progression of these diseases, chronic inflammation has been highly implicated; in fact, it has been considered as the hallmark for pulmonary carcinogenesis [62, 63]. Also, exposure to environmental particles might aggravate infections due to impaired clearance of bacteria [64, 65] and possibly viruses [66].

Cardiovascular Effects

Relatively low PM exposures have been linked to cardiovascular effects, witnessed by a considerable database of published studies reporting associations. The role of PM in cardiovascular illness and its association with heart attacks, stroke and heart rhythm disturbances have been demonstrated in recent years. In acute and chronic studies, PM_{10} exposure has been related to mortality and hospitalisation due to cardiovascular causes [67]. The American Heart Association (AHA) first issued a statement on the cardiovascular hazard of ambient particles in 2004 [68, 69]. The contribution of PM to cardiovascular morbidity and mortality was recognised. In their recent update, the AHA has linked adverse cardiovascular events to $PM_{2.5}$ specifically [70]. Several novel conclusions have been presented: short-term $PM_{2.5}$ exposure (hours to weeks) can already trigger mortality due to cardiovascular causes as well as non-fatal adverse effects. Long-term exposure (e.g. years) increases this risk and reduces life expectancy in highly exposed individuals with months to years. Finally, reductions in ambient PM levels are associated with rapid decreases (i.e. within a few years) in cardiovascular mortality [70].

Chronic PM exposure has been shown to cause increased/accelerated development of atherosclerosis [68, 71]. In young adults exposed to several episodic $PM_{2.5}$ surges, a reduction of circulating endothelial progenitor cells (representing a measure for vascular injury) was found, while inductions of plasma levels of platelet–monocyte aggregates, high-density lipoprotein and non-albumin protein were observed [72]. Concentrations of ambient ultrafine particles and accumulation mode particles have been associated with decreased platelet counts and increased concentrations of plasma sCD40L, a marker for platelet activation, in patients with coronary heart disease [73]. PM_1 inhalation caused impaired vascular function in healthy individuals during exercise, as indicated by reduced flow-mediated brachial artery dilatation and forearm muscle reperfusion dilation as well as basal brachial artery vasoconstriction [74]. A correlation between reactive hyperaemia and the PM_{10} and $PM_{2.5}$ levels was found in a French study [75]. An augmentation of the carotid intima-media thickness, a measure for atherosclerosis, of 5.9% has been found for each 10 $\mu g/m^3$ increase of the ambient $PM_{2.5}$ level in Los Angeles, with even stronger effects reported for women over 60 years of age [76]. Inductions of endothelial dysfunction and ventricular ischaemia have been measured in human volunteers acutely exposed to diluted diesel engine exhaust [77, 78].

A group of people susceptible to cardiovascular effects of PM are diabetes patients. Ambient $PM_{2.5}$ levels have been associated with enhanced expression of markers of systemic inflammation such as C-reactive protein and IL-6 [79, 80] and ventricular repolarisation in diabetic individuals [80]. Two studies from 2007 found associations between levels of ambient particulate matter and various markers of systemic inflammation, oxidative stress and impaired vascular function in diabetic individuals, providing further hints for this potentially sensitive group of people. Associations between $PM_{2.5}$ and black carbon levels and plasma concentrations of the adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) as well as the blood glycoprotein von Willebrand factor (vWF) were found in type 2 diabetes patients [81], while PM_{10} levels have been correlated to higher blood pressure, flow-mediated vasodilatation and systemic oxidative stress [82]. Prothrombotic changes in platelet function and higher amounts of blood leukocytes indicative of systemic inflammation have been observed in diabetic patients, in association with PM_{10} exposure [83].

To investigate the link between exposure to environmental particulates and cardiovascular disease, animal experimental studies have provided more detailed information. Reduced circulating endothelial progenitor cells were measured in CAP-exposed mice [72]. Inhalation of TiO_2 nanoparticles caused impaired endothelium-dependent vasodilation in rat subepicardial arterioles [84]. In ROFA-exposed rats, attenuated arteriole dilation induced by a Ca^{2+} ionophore was measured. Leukocyte adhesion and rolling were induced, suggesting an inflammatory response at the systemic microvascular level, even in the absence of detectable pulmonary inflammation [85]. In a similar study, ROFA exposure was also found to induce adhesion of polymorphonuclear neutrophils (PMN) to the rat microvasculature [86]. A correlation between systemic inflammation and endothelial dysfunction was found in rabbits after acute and chronic PM_{10} exposure [87]. Induction of

enhanced thrombus formation and platelet activation was measured in hamsters exposed to DEP [88, 89]. In mice, reduced platelet counts and tail bleeding time [90] as well as augmented myocardial oxidative stress [91] were measured after DEP exposure. Moreover, platelet pro-aggregatory effects were detected in pial cerebral microvessels [90]. Exposure to ultrafine PM caused a twofold increase in myocardial infarction size and augments post-ischemic oxidative stress in mice. In addition, platelet numbers, plasma fibrinogen and soluble P-selectin levels are increased while bleeding time is reduced, indicative of enhanced thrombogenicity [92]. In another study, spinotrapezius muscle preparations obtained from rats exposed to fine and ultrafine TiO_2 particles were used to evaluate microvascular function. Intraluminal infusion of a Ca^{2+} ionophore caused arteriolar dilations in control animals, which was blunted in animals exposed to TiO_2, especially ultrafine TiO_2 [93].

Attempts have also been made to mimic adverse effects occurring in susceptible individuals, highly relevant for PM-associated mortality and morbidity, employing specific animal models. These include apolipoprotein E-deficient ($ApoE^{-/-}$) and lipoprotein receptor-deficient ($LDLr^{-/-}$) mice, Watanabe heritable hyperlipidemic (WHHL) rabbits and spontaneously hypertensive rats (SHR), which are prone to development of adverse cardiovascular conditions. Enhanced serum VCAM-1 levels, phenylephrine (PE)-induced vasoconstriction and plaque exacerbation were found in CAP-exposed $ApoE^{-/-}$ mice [94]. After DEP exposure, a decrease in acetylcholine-induced vasorelaxation was measured in $ApoE^{-/-}$ mice [95]. In $ApoE^{-/-}$ and $ApoE^{-/-}/LDLr^{-/-}$ single- and double-knockout mice, respectively, subchronic CAP exposure enhances atherogenesis [96]. In $PM_{2.5}$ CAP-exposed $ApoE^{-/-}$ mice kept on a high-fat diet, a highly significant augmentation of composite plaque area was measured compared to air-exposed controls. In animals fed with normal chow, the effect on plaque size was smaller but still reached statistical significance [97]. The lipid content in the aortic arch was 50% higher in the $PM_{2.5}$-exposed group fed with high-fat chow, while in this group, the aortic vasoconstrictor response to challenges with serotonin and phenylephrine was exaggerated and relaxation induced by acetylcholine was attenuated [97]. A more recent study corroborated these findings, with the exception of phenylephrine-induced constriction, which was reduced. Vascular relaxation was attenuated in $PM_{2.5}$-exposed animals compared to controls, while aortic superoxide generation, protein nitration and expression of the NADPH oxidase subunits $p47^{phox}$ and rac1 were induced. Moreover, larger plaques were found in the thoracic aorta, in association with lipid deposition and distinct infiltration by macrophages [98]. In the WHHL rabbit model, featuring naturally occurring atherosclerosis, PM_{10} exposure was also shown to cause amplified monocyte release from bone marrow [99]. Moreover, PM_{10} exposure was shown to cause augmented progression of atherosclerotic lesions in the aortas of hyperlipidemic rabbits and enhanced expression of the adhesion molecules ICAM-1 and VCAM-1 within these plaques. Importantly, circulating monocytes were shown to be recruited into atherosclerotic plaques, which might be an important mechanistic step in the progression of atherosclerosis observed after PM exposure [100]. After exposure to CAP, dose-dependent

increases of white blood cell counts in peripheral blood as well as neutrophil numbers and IL-6 in the bronchoalveolar lavage fluid (BALF) obtained from spontaneously hypertensive (SH) rats were measured [101]. In SH rats exposed to ROFA for 1, 2 and 4 weeks, higher levels of plasma fibrinogen were measured after 1 week only, suggesting that fly ash elicits an acute systemic thrombogenic response [102]. In rats with ozone-induced pulmonary inflammation, an additional exposure to a standardised PM sample (EHC-93 from Ottawa) resulted in further enhanced expression of the pro-inflammatory cytokines tumour necrosis factor-α (TNFα) and macrophage inflammatory protein-2 (MIP-2) in BALF. Additionally, a number of haematological/haemodynamic factors were found to be affected in blood plasma obtained from these rats including endothelin-1 (ET-1) and fibrinogen [103].

Proposed Mechanisms Implicated in the Cardiovascular Effects of PM

Several pathways have currently been proposed to explain for the cardiovascular effects of inhaled particles, as schematically shown in Fig. 2. It has been suggested that cells of the cardiovascular system may be targeted directly by particles (or their constituents) upon their translocation from the lung as well as indirectly by

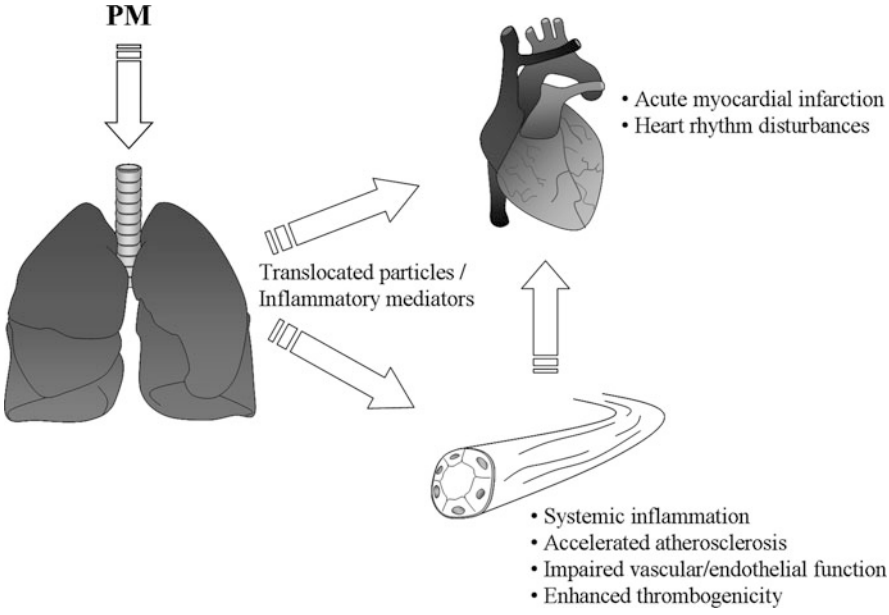


Fig. 2 Particulate matter (PM)-associated cardiovascular effects

inflammatory mediators from the particle-exposed lung tissue. Moreover, PM exposure has been shown to exert alterations in the autonomic balance [68]. These pathways can trigger secondary effects such as arterial vasoconstriction, increased blood pressure, endothelial dysfunction, ST-segment depression, arrhythmias and procoagulant/thrombotic actions.

Nemmar and colleagues have reported that nanoparticles rapidly translocate into the circulation in experimental animals [104] and humans [105]. The findings in hamsters, reported by Nemmar *et al.* [104], have been confirmed more recently in rats [106–108]. However, the occurrence of extrapulmonary translocation in humans has been debated by others [109–111], spawning a controversy that has not been fully resolved. Upon inhalation of carbonaceous nanoparticles, a retention as high as 99% was found in the lungs of humans after 46 h [111]. Although only minute amounts of UFP might translocate into the cardiovascular system in humans, these small amounts could still have adverse consequences for human health due to their large surface area and potential accumulation in chronic exposure settings. Upon entering the circulation, nanoparticles may adversely affect the endothelium as well as the formation of atherosclerotic plaques, especially in susceptible individuals [21, 112]. Also, inflammatory mediators released in response to lung injury can enter the bloodstream and have effects in secondary organs. Adverse effects on the cardiovascular system can easily be induced by mild systemic oxidative stress or pulmonary inflammation in patients with severe cardiovascular disease [113]. Both processes, i.e. systemic particle translocation and pulmonary inflammation, have been considered to explain for systemic inflammatory responses, characterised by the increased presence of (activated) inflammatory cells and mediators as well as acute phase proteins in the circulation observed in PM-exposed humans (e.g. [73, 114–118]).

Neurological Effects

In recent years, the possibility of particle-elicited effects on the central nervous system has been generating increasing attention. CNS pathology has been associated with higher particulate air pollution levels in several studies. Oikonen *et al.* have reported a correlation between ambient PM_{10} levels and multiple sclerosis relapse; in the highest exposure quartile, the risk for the onset of relapse was increased more than fourfold [119]. Another study found enhanced Alzheimer-like pathology in brains from inhabitants of highly polluted cities compared to those residing in cities with low levels of air pollution. Augmented expression of cyclooxygenase-2 (COX-2) and β-amyloid (Aβ42) was measured in the frontal cortex, hippocampus and olfactory bulb from citizens with residence in polluted cities, which are markers for inflammation and progress of Alzheimer's disease, respectively [120]. These and other investigations have sparked toxicologic interest in possible effects of PM on adverse CNS effects. Indeed, in rats, ultrafine carbonaceous particles have been shown to translocate to and accumulate in the brain [121].

Aside from the systemic translocation across the lung–blood barrier discussed before, the involvement of an additional brain-specific translocation route via the nasal epithelium and olfactory nerve was shown for manganese oxide nanoparticles [122].

Several human studies have provided support for the hypothesis that PM adversely affects the CNS and may contribute to diseases such as Alzheimer's disease, Parkinson's disease and multiple sclerosis. Diesel engine exhaust inhalation caused a rapid induction of brain activity in the frontal cortex of human volunteers, suggesting the presence of a cortical stress response [123]. In a cohort of elderly women, exposure to traffic-related PM was associated with mild cognitive function impairments [124]. Concentrations of black carbon in ambient air have been associated with cognitive impairment in children [125]. Moreover, children from Mexico City, notorious for its high levels of air pollution, exhibited cognitive deficits compared to children from a less polluted city, while magnetic resonance imaging showed the presence of hyperintense lesions in the prefrontal cortex [126].

In mice, several CAP inhalation studies have been performed in relation to CNS effects. CAP inhalation resulted in enhanced levels of the pro-inflammatory cytokines IL-1α and TNFα, as well as increased activation of the transcription factor nuclear factor κB (NF-κB) in brain tissue of BALB/c mice [127]. A potential sensitive model to study adverse effects in the brain could be represented by the atherosclerosis-prone $ApoE^{-/-}$ mice, which are additionally susceptible to the development of neuroinflammation and feature enhanced oxidative stress in the brain. In these mice, enhanced activation of NF-κB and activator protein 1 (AP-1) and augmented levels of TNFα and IL-1β were detected after exposure to CAP [128], indicating the induction of inflammatory effects by particles. Moreover, reduced numbers of dopaminergic neurons were found in the substantia nigral nucleus compacta compared to air-exposed control animals [129]. These neurons are a specific target in Parkinson's disease. Augmented concentrations of norepinephrine were found in the paraventricular nucleus of the rat brain after CAP exposure compared to control rats, indicating activation of the stress axis in these animals [130]. Recent results obtained from acute and chronic inhalation studies using diesel engine exhaust suggest region-specific inflammatory and oxidative stress responses in the rat brain [131, 132]. Interestingly, prenatal exposure to diesel engine exhaust has been reported to lead to behavioural changes in mice, indicated by decreased spontaneous locomotor activity [133].

Taken together, current findings suggest that PM exposure might lead to the induction of oxidative stress and inflammation in the brain, processes that are reported to be significantly involved in brain pathology. In parallel, findings on associations of PM exposure with cognitive impairment in children and the elderly, as well as behavioural changes in laboratory animals, have been published. It is not yet fully established, however, whether UFP can translocate to the human brain to elicit effects and a relative contribution of the two possible translocation routes (lung or olfactory route) still needs to be clarified. Also, as of yet, it is unknown whether potential effects can be attributed to a direct effect of translocated particles,

or whether similarly to cardiovascular effect of PM (see Fig. 2), indirect effects driven by pulmonary inflammation may be responsible. Systemic inflammation alone has been known to elicit adverse effects in the brain [134, 135].

Particulate Matter: Oxidative Stress and Inflammation

The Oxidative Stress Paradigm

The main current paradigm in particle toxicology is centred on the concept of oxidative stress, as the ability of particles to generate reactive oxygen species (ROS) is suggested to be the main factor involved in their pathogenic potential. Indeed, there is abundant evidence for the role of oxidative stress in particle-associated adverse effects (e.g. [31, 136–140]).

Oxidants, with oxygen being the prime example, are substances that readily transfer oxygen atoms or accept electrons and by doing so are capable of causing adverse effects such as lipid peroxidation, oxidative DNA damage and protein oxidation. To avoid possible detrimental effects that can result from increased oxidant levels, their counterparts, the antioxidants, serve to control their presence. The term "oxidative stress" was coined by Sies and is defined as the adverse condition resulting from an imbalance in cellular oxidants and antioxidants, with the scale tipping towards the side of the former [141]. For homeostasis to degenerate into a situation of oxidative stress, the amount of reactive species formed needs to be sufficient to overcome and not merely dent the antioxidant shield. The magnitude of the oxidative challenge determines the cellular reaction. Small amounts of ROS are required for processes such as intracellular signalling. When substantial amounts are generated and oxidative stress is induced in response to particle exposure, a hierarchical response has been forwarded by Nel and co-workers [139]. As the amount of ROS increases, the response can evolve from an initial upregulation of cellular antioxidants such as heme oxygenase-1 (HO-1) and catalase to a pro-inflammatory response indicated by activation of factors such as NF-κB and, finally, to toxicity and cell death.

There is abundant evidence that oxidative stress is the responsible factor involved in the rise of pulmonary pathology in response to PM [31, 136, 137]. PM-derived ROS have been implicated in the activation of mitogen-activated protein kinase (MAPK) family members and activation of transcription factors such as NF-κB and AP-1. These signalling pathways have been implicated in processes of inflammation, apoptosis, proliferation, transformation and differentiation [31]. However, the situation is complex, as PM represents a mixture of many different components that consists of a variable particle core and a large array of surface-bound constituents including PAHs and metals. Both the core particles and their constituents are relevant for PM-elicited effects [17, 142, 143]. PM characteristics and components implicated in the generation of ROS include surface area,

transition metals and organic constituents such as PAHs [17, 29, 35, 143–148]. Formation of hydroxyl radicals (•OH) via Fenton chemistry is widely considered to be responsible for the transition metal effect [143, 149]. Particle surface-coated organics can lead to ROS formation via quinone redox cycling [150]. This involves the one-electron reduction of quinones by NADPH cytochrome P450 reductase, resulting in superoxide ($\bullet O_2^-$) generation [151]. PAHs can be converted to quinones within the lung by biotransformation enzymes such as cytochrome P4501A1, epoxide hydrolase and dihydrodiol dehydrogenase [152, 153]. Metals and organic PM components can synergise, leading to exaggerated ROS generation [19].

Aside from direct generation by the particles or their constituents, ROS can be generated by target cells such as lung epithelial cells and pulmonary macrophages upon interaction with and/or uptake of particulate material. In particular, phagocytic cells of the innate immune system such as alveolar macrophages (AM) and polymorphonuclear neutrophils (PMN) are highly proficient producers of ROS to enhance microbicidal conditions in phagocytic vacuoles and eliminate pathogenic bacteria or potentially harmful particles [154–156]. Phagocyte-generated reactive species include superoxide ($\bullet O_2^-$), nitric oxide (•NO), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), peroxynitrite ($ONOO^-$) and hypochlorous acid (HOCl) [62, 157]. Herein, the primary product $\bullet O_2^-$ can be dismutated by superoxide dismutase (SOD) enzymes, upon which H_2O_2 is formed [158], which in the presence of transition metals can give rise to the extremely reactive •OH radical [159]. Additionally, myeloperoxidase (MPO), an enzyme predominantly expressed by PMN, can produce the highly toxic product HOCl from H_2O_2 and chloride anions [160, 161]. Another phagocyte anti-microbial system is the enzyme inducible nitric oxide synthase (iNOS), which generates •NO radicals from L-arginine, NADPH and oxygen. The principal cellular source of •NO in the lung is the macrophage, with neutrophils representing another source. Also, as iNOS is expressed in airway epithelium, lung epithelial cells release •NO in response to inflammatory stimuli such as endotoxin and cytokines [162, 163]. Superoxide formed by NADPH oxidase (NOX) can react with •NO formed by iNOS to produce $ONOO^-$ radicals [160].

Although ROS/reactive nitrogen species (RNS) production is a crucial event in host defence, they are capable of causing damage to cellular macromolecules such as nucleic acids, lipids and proteins [164, 165]. In a chronic inflammatory setting, persistent production of ROS can cause considerable tissue damage. Therefore, people suffering from chronic inflammatory diseases, especially those localised to or affecting the pulmonary region, may be susceptible for adverse health effects of PM inhalation.

ROS are believed to be a major factor in genotoxicity, so the particle characteristics described before as important contributors to ROS formation should also be considered as relevant for their genotoxic potential. It is well known that ROS are capable of causing DNA strand breaks and DNA oxidation, factors implicated in the initiation stage of carcinogenesis [166]. One of the major products of oxidative DNA damage is the premutagenic lesion 8-hydroxy-2′-deoxyguanosine (8-OHdG) [166]. Several *in vitro* studies have demonstrated that PM induces DNA damage in

the form of strand breaks and 8-OHdG formation in lung epithelial cells. These will be discussed in detail in a later section of this chapter. Within the scientific community, there is also general agreement that chronic inflammation and the associated excessive formation of ROS are crucially involved in particle exposure-associated genotoxicity, especially for poorly soluble dusts of low toxicity such as carbon black and titanium dioxide [167]. In various co-culture models, the DNA-damaging potential of activated monocytes/macrophages [168–170] and neutrophils [170–174] was demonstrated *in vitro*.

Taken together, these findings illustrate the notion that inflammation and oxidative stress are intimately linked phenomena. On the one hand, the pro-oxidative potential of a certain particle determines its ability to activate immune cells to secrete pro-inflammatory mediators; on the other hand, inflammation is associated with increased ROS generation by phagocytes, which can contribute to oxidative stress elicited upon PM exposure.

PM-Induced Inflammatory Effects: NF-κB Activation

Whereas the acute inflammatory cascade is generally beneficial, degeneration into a chronic response is of instrumental importance in the development of pulmonary diseases such as COPD, asthma and lung cancer. In addition, cardiovascular disease and atherosclerosis contain a strong inflammatory component. If inhaled particles are reactive, at a sufficiently high dose, the acute inflammatory cascade can be activated. A strong particulate challenge (depending on dose, solubility, particle shape and reactivity) can stimulate AM to secrete a variety of mediators such as chemokines and cytokines. These attract inflammatory cells to the lung, mainly PMN, but also further monocytes/macrophages are recruited. In case of a high acute or chronic exposure to reactive particles, macrophage clearance can be compromised due to particle toxicity. Then, the acute inflammatory response can develop into a state of persistent inflammation.

Among the wide variety of macrophage products released, IL-8 and its murine equivalent MIP-2 are considered as some of the most potent neutrophil chemoattractants [175, 176], while TNFα is a powerful priming agent for neutrophils [177, 178] and IL-1β contributes to leukocytosis by inducing release of neutrophils from bone marrow and elicits the production of other cytokines from a variety of cells [179].

The transcription factor NF-κB functions as the pivotal regulator of inflammatory processes, including those occurring in the lung [180, 181], switching on transcription of a large array of inflammatory genes upon activation. The NF-κB dimer, typically consisting of a RelA/p65 subunit and a p50 subunit (this specific dimer is often referred to as "NF-κB"), resides in the cytosol bound to its IκB inhibitor protein (in most cases IκBα) under normal conditions. The interaction of NF-κB with IκB prevents DNA binding as the latter possesses a strong nuclear export signal and blocks the nuclear localisation signal of NF-κB. The classical

NF-κB activation cascade is shown in Fig. 3. In the canonical pathway, the main route for pro-inflammatory activation, when an inflammatory stimulus reaches the cell, IκBα is phosphorylated at serines 32 and 36 by the IκB kinase (IKK) enzyme complex, ubiquitinated at lysines 21 and 22 by β-transducin repeat-containing protein E3 (β-TrCP E3) ubiquitin ligase and subsequently degraded by the 26S proteasome. The dissociation allows NF-κB to migrate into the nucleus, bind to the so-called κB sites of the DNA and induce the transcription of pro-inflammatory genes such as iNOS [182], COX-2 [183], TNFα [184, 185], IL-1β [186], IL-6 [187], IL-8 and MIP-2 [175, 188], ICAM-1 [189], granulocyte macrophage colony-stimulating factor (GM-CSF, [190]) and many more.

A plethora of NF-κB-activating factors are known, including viruses, bacteria and fungi as well as their products, inflammatory cytokines and their receptors, mitogens, hormones, ROS and RNS and many types of particles. Studies reporting PM-elicited NF-κB activation are summarised in Table 4. NF-κB activation in response to model particles is shown in Table 5. An alternative mechanism for NF-κB activation by PM could be an indirect route via inflammatory mediators (e.g. TNFα, IL-1β) upon their secretion by immune cells, which are known to be highly potent activators of the NF-κB cascade [191, 192]. Conditioned media from PM_{10}-treated macrophages have been shown to induce NF-κB activation in A549 cells [193, 194]. To make matters more complicated, there is evidence for interplay

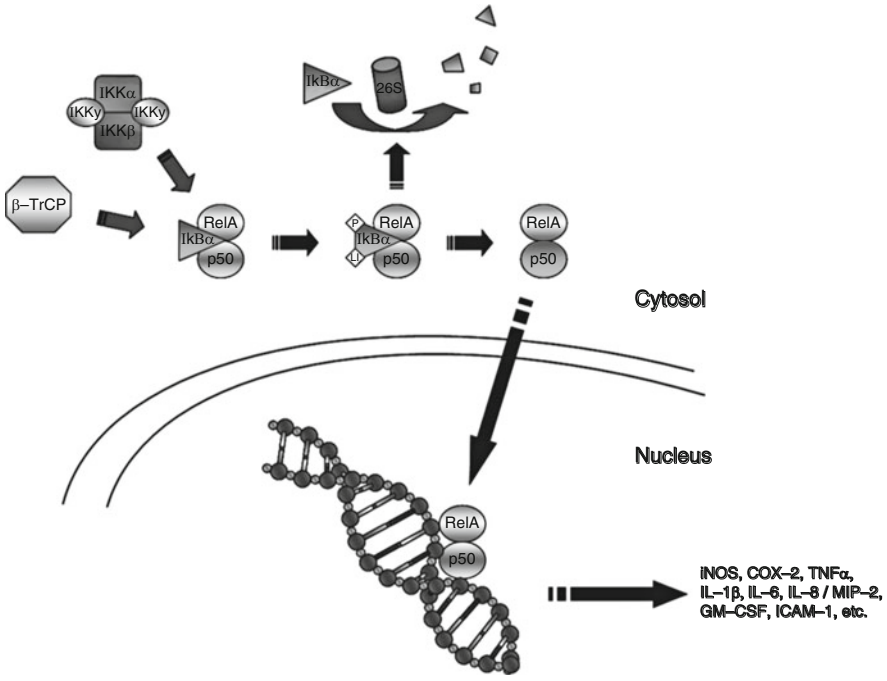


Fig. 3 Classical/canonical pathway of NF-κB activation

Table 4 NF-κB activation by particulate matter (PM)

Particle type	Used model	Target organ	References
$PM_{2.5}$, PM_{10} (Mexico City)	Human umbilical vein endothelial cells (HUVEC)	Cardiovascular system	[324]
$PM_{2.5}$	Human umbilical vein endothelial cells (HUVEC)	Cardiovascular system	[282]
PM (Baltimore)	Human bronchial epithelial cells (HBEpCs)	Lung	[325]
Concentrated ambient particles (CAP) (Los Angeles, CA)	$ApoE^{-/-}$ mice	Brain	[128]
$PM_{2.5}$ CAP (Tuxedo, NY)	Human bronchial epithelial cells	Lung	[295]
$PM_{2.5}$ (Dunkerque)	Human lung epithelial cells (L132)	Lung	[326]
CAP	Ovalbumin-sensitised BALB/c mice	Brain	[127]
PM (Ottawa), iron-loaded fine TiO_2	Rat tracheal explants	Lung	[327]
CAP $PM_{2.5}$	Human bronchial epithelial cells	Lung	[328]
$PM_{2.5}$	Human lung epithelial cells (A549)	Lung	[329]
$PM_{2.5}$	Murine C10 alveolar epithelial cells, NF-κB-luciferase reporter cell line	Lung	[330]

between ROS and cytokine-induced NF-κB activation [195], another crossroad of inflammation and oxidative stress.

Particulate Matter: Cellular and Molecular Targets

The human lung consists of around 40–50 cell types [196], including at least six types of epithelial cells alone, making the lung one of the organ systems with the greatest cellular variety. In addition, effects on cellular organelles and biomolecules might be differential. Recent studies have shown that inhaled $PM_{2.5}$ triggers endoplasmic reticulum stress in cells obtained from the mouse lung and liver [17, 197]. UFP have been found to localise inside damaged mitochondria [17]. Combined with the notion that UFP are capable of negotiating the lung–blood barrier, this provides a staggering amount of possible interactions between inhaled particles and cells. Aggregation and agglomeration of particles and the formation of a protein corona are likely to influence the interaction of particles with biological cells, structures and biomolecules [198]. Coating with opsonins such as antibodies or complement proteins can facilitate phagocytosis by resident immune cells. Depending on the cell type that comes into direct contact with PM upon inhalation, different responses might be expected.

In the following sections, investigations of the effects of PM on specific cell types are discussed in detail. The most relevant cell types for acute inflammatory effects are considered to be pulmonary macrophages and epithelial type II cells. In relation to the cardiovascular effects of PM and UFP, effects on endothelial and

Table 5 NF-κB activation by model particles

Particle type	Used model	Target organ	References
Ultrafine combustion-derived particles (dynamometer)	Human aortic endothelial cells (HAEC)	Cardiovascular system	[331]
Diesel engine exhaust particles (DEP)	Human airway epithelial cells	Lung	[332]
Ultrafine carbon black	Primary rat epithelial lung cells	Lung	[333]
Elemental carbon ultrafine particles	Ovalbumin-sensitised mice	Lung	[334]
DEP	HL-60 cells, differentiated into eosinophils	Lung	[335]
Carbon black (fine, ultrafine, benzo[*a*]-pyrene-coated)	Human lung epithelial cells (A549)	Lung	[336]
Motorcycle exhaust particles	Human lung epithelial cells (A549)	Lung	[337]
DEP	Rat lung epithelial cells (L2)	Lung	[338]
DEP	Mouse epidermal cells (JB6 P(+))	Skin	[339]
Residual oil fly ash (ROFA)	Primary rat airway cells	Lung	[340]
DEP	Human bronchial epithelial cells (BTE-1), primary human airway epithelial cells	Lung	[341]
Ultrafine iron particles	Sprague Dawley rats	Lung	[342]
Ultrafine soot and iron particles (combination exposure)	Rats	Lung	[343]
ROFA	Perfused rabbit lung	Lung	[344]
DEP	Human bronchial epithelial cells (16HBE)	Lung	[304]
DEP, organic extracts of DEP	Human bronchial epithelial cells (16HBE)	Lung	[34]
Organic extracts of DEP	Human bronchial epithelial cells (BEAS-2B), primary human airway epithelial cells	Lung	[345]
ROFA	Human monocytes	Lung, cardiovascular system	[213]
DEP	Human bronchial epithelial cells	Lung	[346]
Soot	Co-cultured human blood monocytes and human bronchial epithelial cells (BEAS-2B)	Lung	[347]
Soot, ultrafine carbon black	Human alveolar macrophages, human blood monocytes	Lung	[348]
Soot, ultrafine carbon black, TiO_2	Human alveolar macrophages	Lung	[349]
ROFA	Human bronchial epithelial cells (BEAS-2B)	Lung	[350]

cardiac cells are presented, while fibroblasts are discussed in relation to fibrotic responses. Dendritic cells are essential cells modulating effects on the adaptive immune system, inducing the allergic response which characterises asthma.

Microglial cells, considered as the macrophages of the CNS and neurons, are included as (potentially) important target cells for adverse neurological effects.

Effects on Macrophages and Monocytes

Traditionally, the main cell type involved in the acute inflammatory response in general and more specifically in response to particles was thought to be the AM, and thus, early research in particle toxicology focused on this cell type (e.g. [199, 200]). Upon deposition in the alveoli, particles are normally cleared by the macrophage population. Several subpopulations exist in the lung; most macrophages are of the alveolar type, but additionally, interstitial and intravascular macrophages are present. They are capable of phagocytosing large amounts of particles, which they attempt to break down by releasing the content of their lysozymes into the phagocytic vesicles containing the particles. However, when particles are poorly soluble and durable, they cannot be disintegrated by macrophages in this way, and instead, fully loaded cells will clear these particles by migrating into the lymphatic system or by exiting the lung via mucociliary clearance, thereby generally entering the digestive system [201, 202]. AM are rapidly replaced by monocytes recruited from the circulation; after maturation, these can develop into fully functional macrophages. AM have been shown to be less capable of recognising UFP, and therefore, their clearance is limited unless individual particles cluster together, forming aggregates or agglomerates. The following major effects of PM on macrophages and monocytes have been reported:

- Activation and release of inflammatory mediators
- Generation of reactive oxygen and nitrogen species
- Impaired functionality and responsiveness to viruses and bacteria
- Role of monocytes in atherogenesis

These effects will be discussed in the next subsections.

Enhanced Release of Inflammatory Mediators

As pivotal cell types in the regulation of acute inflammatory processes, macrophages are capable of releasing a large array of pro-inflammatory mediators. Since inflammation is highly involved in PM-induced adverse effects, pro-inflammatory activation of AM in response to ambient particulates has been well investigated in primary cells as well as in cell lines from humans and rodents. Studies addressing the inflammatory effects of PM have also been performed using co-cultures of macrophages, monocytes, epithelial cells and/or dendritic cells. A summary of the major findings in studies addressing the inflammatory mediator release by AM in response to PM and related model particles is provided in Table 6. In various studies, it could be shown that PM and DEP activate these cells for the production

Table 6 Inflammatory mediators expressed and/or released from PM-treated macrophages

Inflammatory mediator	Cells	Particles	References
TNFα	Human monocytes and alveolar macrophages (AM), monocyte-derived macrophages, THP-1 and U937 human monocytic/ macrophage cell lines, RAW246.7 mouse macrophages	PM_{10}, DEP, traffic-related particles, EHC-93, ultrafine carbon black	[193, 203, 205, 206, 211, 214–218]
IL-1β	Human AM, THP-1, human monocytes	$PM_{10,}$ traffic-related particles, EHC-93, PM, volcanic dust, ROFA	[193, 205–208, 239]
IL-6	Human monocytes/AM, monocyte-derived macrophages, U937, RAW246.7	PM_{10}, DEP, EHC-93, PM, volcanic dust, ROFA	[193, 203, 205, 207, 208, 211, 214, 215, 217, 218, 239]
IL-8	Human monocyte-derived macrophages and AM, THP-1, U937	PM_{10}, DEP, traffic-related particles, EHC-93	[193, 205, 206, 211]
IL-10	Human monocyte-derived macrophages	PM_{10}, DEP	[203]
GM-CSF	Human AM	$PM_{10,}$ EHC-93	[193, 205]
MCP-1	Human AM	Coarse PM	[217]
COX-2	Human Mono Mac 6 cell line, U937 cells, primary blood mononuclear cells, monocyte-derived macrophages	PM, DEP	[210, 211, 217, 218]
PGE_2, LTB4	U937 human macrophage cell line, human Mono Mac 6 cells, primary blood mononuclear cells, monocyte-derived macrophages	Carbon black	[212]

of inflammatory cytokines including TNFα, IL-1β, GM-CSF, IL-6 and IL-8 [193, 203–206]. Statins, anti-inflammatory substances known for their beneficial effects in cardiovascular disease, have been shown to attenuate these pro-inflammatory responses [205]. In PM_{10}-treated human monocytes and mouse macrophages (the J774 cell line), increased concentrations of intracellular calcium, indicative of cellular activation, were measured [207]. The enhanced TNFα release and IL-1α expression in PM_{10}-treated monocytes could be inhibited by calcium antagonists, suggesting a role for intracellular calcium signalling in PM_{10}-induced pro-inflammatory activation of macrophages [207, 208]. In primary macrophages obtained from individuals exposed to coarse PM, enhanced TNFα mRNA expression and upregulation of mCD14, CD11b and HLA-DR have been measured on the cell surface in association with enhanced phagocytosis [209]. PM, DEP or carbon black

particles have also been shown to prime or activate macrophages for enhanced COX-2 expression and prostaglandin E_2 (PGE_2) release in response to LPS [210, 211]. Remarkably, COX-2 expression could be inhibited by an aryl hydrocarbon receptor (AhR) antagonist [211]. Several types of carbon particles elicited augmented generation of arachidonic acid (elemental carbon, Printex 90, Printex G and DEP) and the arachidonic acid (AA) metabolites PGE_2 (elemental carbon, Printex 90, Printex G and DEP) and leukotriene B4 (elemental carbon) by canine and human AM [212]. The effects depended on mitogen-activated protein kinase kinase 1 (MAPKK1)-induced activation of cytosolic phospholipase A_2 ($cPLA_2$) [212]. In ROFA-treated human AM, the JNK and p38 MAP kinases were activated, while neither NF-κB activation nor IL-8 production was detected. In contrast, in human monocytes JNK, p38 and ERK were activated along with NF-κB, while IL-8 production was enhanced [213]. Treatment of murine RAW246.7 macrophages with coarse and fine PM resulted in enhanced release of arachidonic acid, TNFα and IL-6. The effects of carbon black treatment were significantly lower, suggesting that components bound to the PM surface play a major role in PM-induced pro-inflammatory activation of macrophages [214, 215]. Enhanced TNFα release from primary rat alveolar macrophages in response to ultrafine carbon black treatment was inhibited by augmentation of cellular glutathione levels [216].

Although the ultrafine fraction of PM is often forwarded as the most dangerous, the hazard posed by other fractions should not be ignored. Studies by Becker and colleagues have identified coarse PM to be a more effective inducer of pro-inflammatory mediator release by human AM than $PM_{2.5}$. The endotoxin content of the particles has been implicated in this effect, as shown by particle treatment with endotoxin inhibitors [217] and specific inhibition of toll-like receptor 4 (TLR4) [218]. A more recent study confirmed the greater pro-inflammatory potential of coarse PM compared to the fine (defined as $PM_{2.5}$-$PM_{0.2}$) and the ultrafine (defined as $PM_{0.2}$) fractions, using RAW246.7 murine macrophages [219]. These *in vitro* findings are also in line with observations in rodent models [13, 220].

Activation of pro-inflammatory mediators by PM has also been demonstrated in various co-culture studies. After PM_{10} treatment, enhanced mRNA levels of GM-CSF, M-CSF, MIP-1β, MCP-1, ICAM-1 and IL-6 were found in co-cultures of human AM and human bronchial epithelial cells (HBEC) compared to non-treated co-cultures or treated monocultures of both cell types. On the protein level, enhanced concentrations of GM-CSF, MIP-1β and IL-6 were detected in PM_{10}-treated co-cultures compared to treated monocultures; these inductions were independent of ICAM-1 as determined by inhibition experiments [221]. Using a co-culture of monocytes and pneumocytes, enhanced release of pro-inflammatory cytokines was detected after treatment with traffic-derived PM. IL-8 secretion was inhibited using TNFα and IL-1β antagonists, while IL-6 release was reduced only by the IL-1β antagonist [222]. In a co-culture of human monocytes and human AM, the expression of surface receptors involved in T-cell interaction was determined. On monocytes, the expression of HLA-DR, CD40, CD80 and CD86 was upregulated after treatment with coarse, fine and ultrafine PM, but not DEP [223].

In contrast, no particle-related effects on surface receptor expression were found in AM; however, only AM released the T-helper lymphocyte chemokine IL-16 in response to the fine PM fraction [223]. Higher TNFα concentrations were measured in a DEP-treated triple co-culture system (A549 cells, monocyte-derived macrophages and monocyte-derived dendritic cells) compared to the sum of concentrations in the three monocultures; however, lower amounts of IL-8 were measured [224].

Enhanced Generation of Reactive Species

To facilitate neutralisation of potentially harmful bacteria and viruses or their products, macrophages are capable of generating reactive species such as ROS and RNS. In many studies, it has been demonstrated that exposure to PM leads to the induction of these reactive components, which can result in damage to cells and tissues. PM was found to augment ROS generation by human monocyte-derived macrophages [225] and rat alveolar macrophages [226], in contrast to carbon black and DEP [225]. In a recent study, $PM_{2.5}$ induced intracellular ROS generation from human monocytes [204]. ROFA treatment rapidly induces ROS production by human monocytes as well as alveolar macrophages [213, 227], which was decreased by tyrosine kinase inhibitors [213]. Treatment of human AM with ROFA, coal fly ash and DEP identified ROFA as the strongest stimulator of ROS release [228]. After DEP treatment, augmented ROS production was measured in an advanced triple cell co-culture consisting of A549 cells, monocyte-derived macrophages and monocyte-derived dendritic cells, as well as in A549 and macrophage monocultures. In addition, a reduction of the antioxidant capacity was found in the triple co-culture compared to the summed capacity of monocultures [224]. Treatment with elemental carbon particles, Printex 90, Printex G and DEP induced enhanced ROS generation by human and canine AM, dependent on MAPKK1-activated cPLA2 [212]. Carbon black particles induce a concentration-dependent increase in ROS release from rat AM; inhibitor experiments showed involvement of the ERK and p38 MAP kinases [229].

Contrasting effects on nitric oxide production by murine macrophages (J774A.1, WR19M.1, RAW264.7 and NR8383 cell lines) have been reported after treatment with different PM samples or PM in the presence of LPS or IFN-γ, whereas addition of urban PM elicited augmented •NO production, and $PM_{2.5}$ treatment resulted in the opposite [230]. Enhanced •NO release has been reported in PM_{10}-treated RAW246.7 mouse macrophages [231], especially for PM sampled during winter [232]. Treatment with fine and coarse PM results in a dose-dependent enhanced •NO release from RAW246.7 macrophages, with the strongest effects observed for fine PM [233]. In contrast, coarse PM, but not fine PM, collected from contrastingly polluted areas induced •NO release from NR8383 macrophages [61].

Impaired Functionality and Responsiveness to Viruses and Bacteria

Epidemiological studies have shown that pneumonia incidence increases after episodes of augmented PM levels. It has been hypothesised that PM interferes with the ability of AM to fulfil their task as primary pulmonary guardians against potentially harmful bacteria and viruses. A number of *in vitro* studies have investigated the influence of PM on macrophage functionality.

The expression of several immune receptors important for bacterial clearance has shown to be downregulated in PM-treated human AM and blood-derived monocytes. Moreover, phagocytosis and oxidant generation were inhibited by PM [234]. Coarse PM was shown to inhibit AM responsiveness to yeast and to elicit apoptosis in these cells, in contrast to $PM_{2.5}$ [217]. Impaired AM responses to respiratory syncytial virus-infected airway epithelial cells were observed when PM_{10} was added to the co-culture system [66]. In human AM, coarse and fine PM as well as DEP inhibited LPS-elicited TNFα release [218]. Decreased ROS generation was observed in rat AM treated with coarse or fine PM and subsequently with the PKC-activator phorbol myristate acetate (PMA), compared to AM treated with PMA only [235]. AM obtained from rats exposed intratracheally to DEP produced more IL-1β compared to those obtained from control animals [236]. However, when treating cells with LPS *ex vivo*, AM from DEP-exposed rats showed decreased TNFα and IL-1β production compared to the summed releases induced by DEP and LPS. Confirming the latter observation, similar results were found using AM from rats exposed to both DEP and LPS *in vivo* [236]. DEP treatment inhibited LPS- and IFN-γ-induced TNFα and IL-6 production by murine AM, and LPS-induced TNFα, IL-6 and IL-8 release by human AM. When AM were pre-treated with superoxide dismutase, these suppressive effects were markedly reduced [237]. J774A.1 macrophages treated with Printex 90 and elemental carbon particles, but not DEP or urban PM, showed enhanced cytoskeletal dysfunction and compromised phagosome transport. Cytoskeletal disturbance was reduced by calcium inhibitors [238]. Treatment with ROFA induced apoptosis in human AM while PM samples caused pro-inflammatory activation; the ability of PM to elicit apoptosis and inflammation is therefore not correlated [239].

Monocytes and Atherogenesis

Monocyte migration into atheromas, where they form foam cells, is one of the hallmarks of atherosclerosis. Interactions between monocytes and endothelial cells have been investigated in a recent *in vitro* study. Treatment with $PM_{2.5}$ elicited enhanced adhesion to aortic endothelial cells [204]. In addition, conditioned media derived from PM-treated pulmonary microvascular endothelial cells caused pro-inflammatory responses in monocytes [204]. An association between particle-elicited pro-inflammatory responses and cholesterol accumulation, one of the main characteristics of foam cells, was reported in DEP- and PM-treated U937 human macrophages [211].

Effects on Lung Epithelial Cells

A considerable number of *in vitro* studies exist in which effects of PM on lung epithelial cells have been investigated. Major observed effects include activation of signalling cascades involved in the activation and release of pro-inflammatory mediators and cell proliferation, and DNA damage responses. Although the earliest/fastest inflammatory activation in the lung has been detected in alveolar macrophages [240], an important role for pulmonary epithelial cells (in particular, bronchial epithelial cells and type II alveolar epithelial cells) as regulators of inflammatory responses in the lung is becoming increasingly clear. In many studies, it could be demonstrated that PM as well as specific size fractions within this mixture can trigger the release of pro-inflammatory mediators from bronchial as well as alveolar epithelial cell lines (see Table 7). The induction of oxidative stress and subsequent activation of NF-κB and MAPK signalling cascades have been considered as the central underlying mechanisms herein [31]. Findings in specific studies also indicate that PM activates cell signalling cascades involved in proliferative responses of epithelial cells. ROS- and oxidative stress-mediated activation of NF-κB and MAPK pathway activation in human lung epithelial cells as well as the associated release of inflammatory mediators such as IL-8 and GM-CSF have been shown for DEP and ultrafine carbon black particles (e.g. [151, 241–244]). As already discussed, PM, as well as DEP and ultrafine carbon black, has also been demonstrated to induce DNA damage, typically measured as strand breaks or the formation of the oxidative lesion 8-OHdG in lung epithelial cells [142, 144, 148, 245–250]. For PM, the importance of ROS and transition metals has been clearly established in several of these studies by the inclusion of antioxidants or metal chelators (e.g. [142, 144]). However, other studies with PM and more specifically with DEP, which is rather low in metal content, also suggest a role for organic constituents in oxidative DNA damage induction via quinone redox cycling [249]. It has been also shown that PM can trigger responses in epithelial cells that may affect cell proliferation. Disturbed homeostasis of the lung epithelium resulting

Table 7 Mediators released from lung epithelial cell lines after treatment with particulate matter (PM)

Effects	PM component	Cells	References
TNFα	$PM_{2.5}$	16-HBE	[146]
IL-6	PM_{10}	A549, BEAS-2B, HBEC	[221, 258, 351]
IL-8	PM_{10}	A549	[61, 258, 260, 262]
IL-1α	$PM_{2.5}$	16-HBE	[146]
GRO-α	$PM_{2.5}$	16-HBE	[256]
CRP	PM_{10}	A549	[352]
Hsp-70	PM_{10}	A549	[352]
Amphiregulin	$PM_{2.5}$	16-HBE	[255–257]
GM-CSF	PM_{10}, $PM_{2.5}$, PM_{1}, $PM_{0.1}$	16-HBE, HBEC	[146, 221, 259, 353]
TGFα	$PM_{2.5}$	16-HBE	[255]
HB-EGF	$PM_{2.5}$	16-HBE	[255]
MIP-1β	PM_{10}	HBEC	[221]

from increased epithelial cell proliferation in response to inhaled particles has been implicated in airway remodelling as well as tumour promotion [251]. $PM_{2.5}$ and ultrafine carbon black have been shown to trigger dose-dependent cell cycle activation and/or apoptosis in C10 mouse alveolar epithelial cells with specific upregulation of the mRNA expression of *jun* and *fos* genes [252]. Ultrafine carbon black has also been shown to induce EGF receptor-mediated proliferation of human primary epithelial cells [253] and RLE-6TN rat lung epithelial cells [254]. Observations in the latter study indicate that the proliferative *versus* the pro-apoptotic responses triggered by the ultrafine carbon black particles depended on their ability to activate β1-integrin-mediated signalling [254]. In $PM_{2.5}$-treated 16-HBE bronchial epithelial cells, an induced mRNA expression and protein release of transforming growth factor-α (TGF-α) and heparin-binding EGF-like growth factor (HB-EGF) have been reported [255]. These effects were mainly attributed to the organics and to a lesser extent to metallic components [255]. In the same cell line, $PM_{2.5}$ has also been shown to induce amphiregulin mRNA and protein expression, considered to play a role in bronchial tissue remodelling [256, 257]. This effect was shown to be dependent on oxidative stress and the induction of ERK MAPK [257]. DEP has also been shown to enhance the expression of the proto-oncogene c-fos in 16HBE human bronchial epithelial cells [242].

When considering the effects of inhaled PM towards epithelial cells, the role of macrophages should also be considered as already discussed in the previous section. In this regard, several *in vitro* studies have been performed in which lung epithelial cells were treated with conditioned media of PM-exposed macrophages. Treatment of A549 cells with supernatants of AM that were pre-treated with PM_{10} were found to activate both NF-κB and AP-1 as well as to induce the mRNA expression of IL-1β, IL-8, leukaemia inhibitory factor (LIF) and regulated on activation normal T cells expressed and secreted (RANTES). Notably, these responses were reduced when supernatants were pre-incubated with anti-IL-1β and anti-TNFα neutralising antibodies [193]. The enhanced release of IL-8 and augmented expression of ICAM-1 in A549 cells induced by supernatants of PM_{10}-treated human monocytes were found to be reduced when the monocytes were simultaneously treated with calcium inhibitors [208].

The responses of epithelial cells towards PM, similar to those in macrophages, have been found to depend on the sampling location (e.g. [258, 259]), sampling season (e.g. [148, 259]) as well as on the size fraction of the materials (e.g. [61, 148]). This again underscores the important role of the physicochemical properties of the particulates. In concordance with studies in macrophages, it has been revealed that the pro-inflammatory potential of PM in epithelial cells may depend on their endotoxin content [258]. However, several studies have demonstrated that transition metals and organic constituents are likely of more importance for epithelial cell responses. The ability of PM to induce NF-κB activation and associated IL-8 release from A549 cells was found to be mediated by iron [260]. The organic component of PM has been demonstrated to cause transactivation of the xenobiotic response element (XRE), leading to increased expression of cytochrome P4501A1 (CYP1A1) and NADPH-quinone oxidoreductase-1 (NQO-1), respectively [259].

Induction of these phase I and phase II metabolism enzymes has also been observed with DEP in relation to its organic component [151].

Finally, investigations with lung epithelial cells have also revealed interesting observations that may explain for susceptibility differences in humans with regard to the adverse health effects of inhaled PM. The activation of the transcription factors NF-κB [261, 262] and AP-1 [262] by PM_{10} was found to be enhanced in adenoviral early region 1A (E1A)-transfected A549 cells in comparison to non-transfected cells. In addition, enhanced IL-8 [261, 262] and ICAM-1 [261] expression has been measured in these transfected cells. Adenoviral early region 1A (E1A) has been associated with enhanced COPD risk, and as such may provide a basis for susceptibility differences towards effects of inhaled PM in healthy individuals *versus* COPD patients. Devalia and colleagues have investigated the responsiveness of primary bronchial epithelial cells from asthmatic and non-asthmatic individuals towards DEP. They found that both baseline and DEP-induced releases of IL-8, GM-CSF and sICAM-1 were greater in the cells from the patients than in those obtained from the non-asthmatic individuals [263].

Effects on Fibroblasts

Various studies have reported effects of PM on fibroblasts. Treatment of primary rat fibroblasts with fly ash has been shown to induce oxidative stress and the activation of the p38 MAPK, p44/32 MAPK and p70 (S6) kinase enzymes. Also, the expression of collagen 1 and TGF-β1 was induced, while TNFα and MMP9 mRNA levels were found to be reduced [264]. At high concentrations, fibroblast proliferation was inhibited [264]. Similar findings were reported for $PM_{2.5}$, which stimulated fibroblast proliferation at low concentrations but inhibited cell growth at high concentrations [265]. Possibly, effects at lower concentrations are more relevant for the real-life situation. Enhanced platelet-derived growth factor (PDGF)-α receptor expression on the surface of lung fibroblasts, which determines the magnitude of the fibroblast response to the potent mitogen PDGF, was elicited by conditioned media from ROFA-treated macrophages [266]. PM_{10} samples from Mexico City induce upregulation of the PDGF-α receptor on the surface of myofibroblasts, both directly and indirectly via soluble macrophage products [267]. In another study, tissue repair by fibroblasts was measured using the collagen gel contraction test. Ultrafine carbon black particles were found to inhibit fibroblast-mediated tissue repair, which was related to the binding of fibronectin and TGF-β to the particle surface [268].

Effects on Dendritic Cells

PM possesses adjuvant activity on allergic airway sensitisation involved in asthma. A number of *in vitro* studies using dendritic cells, antigen-presenting cells

considered to be crucial for the adaptive immune response in general and the initiation and maintenance of airway immune responses in Th2 type allergic diseases (e.g. asthma) more specifically, provide mechanistic hints for this effect. Most of the studies have been carried out with DEP. Dendritic cells exert phagocytic activity and can thus internalise PM, although this activity is lower than that of monocytes or macrophages and is partly lost upon maturation [269]. DEP treatment of dendritic cells has been shown to induce the production of TNFα, IL-6, IFN-γ, IL-12 and vascular endothelial growth factor (VEGF). Cellular activation has also been measured as antigen uptake and the expression of MHC class II and CD40 on the cell surface [270]. In co-culture with CD4+ T cells, a Th2-like pattern of cytokine production has been observed, characterised by the enhanced production of IL-13, IL-18 and reduced IFN-γ production. CD4+ T cells were not functionally activated by DEP in a monoculture [270]. Treatment of DC with PM has been reported to result in a differential modulation of their activation. Enhanced expression of co-stimulatory receptors, but blunted expression of the endocytosis receptor CD206 and toll-like receptors 2 and 4 was observed. Whereas secretion of IL-12 and IL-6 was decreased, secretion of IL-10 and IL-18 was augmented. When cultured with CD4+ T cells, a Th2-like pattern of cytokine production was measured upon PM treatment, contrasting with the Th1-polarising effects observed after LPS treatment of DC [271]. In some studies, DEP has also been shown to inhibit DC cell maturation. After combined DEP and LPS challenge of immature murine bone marrow-derived dendritic cells, lower IL-12p70 production was found; additionally, these DC lost their ability to suppress IgE isotype switch in B cells, indicating lower functionality [272]. Similar observations were made in a study where DC were treated with organic extracts from DEP. Expression of maturation receptors and IL-12 production elicited by LPS were inhibited [273]. These findings suggest that DEP and specifically DEP-bound organics can interfere with the induction of Th1-promoting response pathways.

PM-associated oxidative stress, attributed to organic constituents, has been hypothesised to be involved in allergic pulmonary inflammation [274]. A role for PM-induced oxidative stress in proallergic Th2-mediated immune responses was found in a study by Williams *et al.* [275]. DEP were found to induce T-cell proliferation *in vitro* [57]. Also, oxidative stress induced by DEP organic extracts was reported to inhibit maturation and IL-12 production via activation of Nrf-2, which inhibits IL-12 production [273]. Although a role for the DEP-bound organics is likely considering the scientific evidence, some evidence suggests that the carbon particle core itself is also involved in affecting DC responses: carbon black particles elicit DC maturation, indicated by enhanced expression of CD80 and CD86 [56]. Another study found increased expression of DEC205 and CD86 in mouse bone marrow-derived dendritic cells treated with ultrafine carbon black [276].

Besides biological consequences of direct particle treatment, indirect effects have been reported mediated by pulmonary epithelial cells, which reside in close contact to DC in the lung. Using immature monocyte-derived DC, no direct induction of maturation was observed after treatment with DEP in a broad concentration range. Conditioned medium obtained from DEP-treated human bronchial

epithelial cells (HBEC), however, induced functional maturation of DC dependent on GM-CSF [277]. Another factor implicated in DEP-induced DC maturation mediated by the lung epithelium is thymic stromal lymphopoietin (TSLP), which is upregulated in asthmatic airways [278]. DEP induce upregulation of TSLP in human bronchial epithelial cells, which elicits dendritic cell maturation and Th2 polarisation [279].

Effects on Endothelial, Cardiac and Smooth Muscle Cells

The ability of PM to elicit oxidative stress in and the production of inflammatory cytokines by endothelial cells has been demonstrated in various studies. In human aortic endothelial cells (HAEC), DEP treatment elicited superoxide generation and the expression of heme oxygenase-1 (HO-1) and tissue factor (TF), indicative of oxidative stress. Also, JNK activation was found to be involved in these effects [280]. At a non-toxic dose, $PM_{0.1}$ was found to induce ROS generation in mouse pulmonary microvascular endothelial cells (MPMVEC), which was supported by *ex vivo* imaging of endothelial cells employing a perfused lung model [281]. $p67^{phox}$ siRNA inhibition experiments and experiments using MPMVEC isolated from $gp91^{phox}$ knockout mice demonstrated the crucial involvement of NADPH oxidase 2 (NOX2) in the observed endothelial ROS generation; additionally, translocation of NOX subunits to the cell membrane was shown [281]. The p38 and ERK1/2 MAPK pathways were activated in MPMVEC in response to $PM_{0.1}$, which was absent in $gp91^{phox-/-}$ MPMVEC and effectively inhibited using $p67^{phox}$ siRNA [281]. Urban PM caused oxidative stress in vascular endothelial cells, with the sources of ROS identified to be NADPH oxidase and the mitochondria [47]. $PM_{2.5}$ elicits oxidative stress, NF-κB activation and cytotoxicity in human umbilical vein endothelial cells [282]. Ultrafine carbon black and urban PM induce release of the pro-inflammatory cytokines IL-6 and IL-1β from primary rat cardiomyocytes and cardiofibroblasts, while TNFα was mainly induced by the PM sample. Moreover, IL-6 induction was augmented after treatment with the conditioned media from particle-exposed primary rat lung epithelial cells [283]. Using primary human vascular endothelial cells, significant increases of IL-6 were found after PM treatment. A further synergistic augmentation of PM-induced IL-6 was found in a co-culture of endothelial cells with human white blood cells [284]. Carbon black treatment induced cytotoxicity and monocyte chemoattractant protein-1 expression in HUVEC cells, while cell growth and the expression of connexion 37 and endothelial nitric oxide synthase (eNOS) were inhibited [285].

Increased expression of the cellular adhesion molecule E-selectin was found on HUVEC stimulated with PM_{10}; moreover, the adhesivity of added monocytes to these cells was highly induced by the particles. The effects were linked to the endotoxin content [286]. Supernatants harvested from DEP-treated human monocyte-derived macrophages strongly induced the expression of the adhesion molecules ICAM-1, VCAM-1 and E-selectin on the surface of endothelial cells. This

induction was mediated by TNFα, while the particulate iron content was suggested to be responsible for macrophage activation [287]. $PM_{2.5}$ and PM_{10} samples from Mexico City were reported to induce adhesion of monocytes to HUVEC cells, in association with enhanced expression of adhesion molecules [288].

Enhanced endothelium permeability permits stronger transmigration of inflammatory cells into subendothelial tissue, which is implicated in atherosclerosis. Increased permeability was observed in human aortic endothelial cells treated with DEP, measured using the horseradish peroxidase–streptavidin activity assay. Also, a downregulation of zonula occludin-1, a tight junction protein, was found. Both effects were more pronounced after treatment with DEP from a diesel engine functioning on an urban dynamometer driving schedule compared to DEP derived from an idling engine [289]. Disturbance of the barrier function in human pulmonary endothelial cells was reported after PM treatment, which was dependent on ROS generation and associated with activation of p38 MAPK and heat shock protein 27 [290].

To investigate vascular smooth muscle function, the ability of PM to modulate relaxation of aortic rings has been assessed. PM significantly induced relaxation of phenylephrine (PE)-precontracted aortic rings in a concentration-dependent manner; ROS, but not soluble iron, were involved in this effect [291]. In another study, enhanced oxidative stress was detected in isolated rat aortic rings upon DEP treatment, which was inhibited using superoxide dismutase (SOD), indicating a role for superoxide [292]. NO production by the NO-generating substance 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide was decreased by DEP, and relaxation induced by acetylcholine and NO-donors was inhibited or attenuated after DEP treatment [292]. The effect of both vasodilators on aortic relaxation was restored by the addition of SOD [292]. These findings suggest that DEP affect the bioavailability of endothelial-produced NO by the induction of superoxide production, leading to impaired vascular function.

Effects on Neurons and Microglial Cells

Some relatively recent *in vitro* studies have demonstrated the ability of particles to affect toxicity in neuronal cells and disturb the normal function of the blood–brain barrier. Selective toxicity of DEP for dopaminergic neurons has been shown in a mesencephalic neuron–glia co-culture. Microglial activation, resulting in the activation of the superoxide-producing NOX enzyme, was critically implicated in this effect [293]. Augmented TNFα levels and enhanced expression and activation of the drug efflux transporter P-glycoprotein were found in rat brain capillaries in response to DEP treatment [294]. Treatment of microglial cells, the activation of which is considered to be highly relevant for oxidative stress-mediated neurodegeneration, with CAP resulted in inflammatory activation [295]. *In vitro* exposure to ultrafine titanium dioxide enhanced microglial activation in cells co-exposed to LPS [296].

Conclusions

Since the identification of the hazard posed by environmental particulates, a large body of scientific data has been generated investigating its nature, extent and mechanisms. Epidemiological studies have provided insight into the risk posed by ambient particles, while animal studies and *in vitro* investigations have contributed to mechanisms and particle components involved in adverse health effects. Classically, toxicological investigations have focused on the lung, while at a later stage, research expanded to extrapulmonary target organs. Due to convincing correlations found in epidemiological studies, the cardiovascular system has been the subject of research in particular, while in very recent years, the central nervous system has come under investigation as a further potential target organ; neurological effects are, however, still under discussion. Due to the high relevance of susceptible populations for PM-related mortality and morbidity, sensitive *in vivo* models have been used to mimic effects occurring in these individuals. The associations found in epidemiological studies can be substantiated by plausible biological mechanisms identified in experimental studies. Importantly, one should also be critical of the interpretation of *in vitro* toxicity studies in relation to the dose applied. When using pulmonary epithelial cells or macrophages as model, it is relatively easy to compare the applied *in vitro* concentrations with deposition estimates in relation to ambient exposure levels and particle properties including size, density and surface area. However, translocation rates of specific components of PM (e.g. ultrafine particles, organics, metal ions) from the cells lining the respiratory tract lumen towards pulmonary endothelial cells or fibroblasts, as well as further into secondary organs and tissues, are poorly investigated. Hence, *in vitro* studies (e.g. with cardiovascular cells or neural cells) should be interpreted with caution. Depending on the concentrations of PM and/or their specific (model) constituents, effects may range from mild oxidative stress and activation of pro-inflammatory signal cascades to mere toxic, inactivating responses. For future *in vitro* studies, also a stronger focus should be directed towards approaches which allow for a better characterisation of interactions between different cell types, i.e. using more advanced co-culture models. This will also allow for the investigation of the indirect effects of mediators released from the respiratory tract towards distal cells and organ systems. Additionally, the evaluation of a broad spectrum of effects is recommended to assess the two main factors in PM-related adverse health effects:

- Oxidative stress is an elusive phenomenon that is difficult to measure directly; therefore, the upregulation of cellular defence responses as well as the exaggerated presence of oxidants should preferably be shown.
- Different particulate materials and components can elicit differential inflammatory mechanisms and pathways; therefore, multiple factors and pathways should be investigated.

References

1. Bowman DM, Balch JK, Artaxo P, Bond WJ, Carlson JM, Cochrane MA, D'Antonio CM, Defries RS, Doyle JC, Harrison SP, Johnston FH, Keeley JE, Krawchuk MA, Kull CA, Marston JB, Moritz MA, Prentice IC, Roos CI, Scott AC, Swetnam TW, van der Werf GR, Pyne SJ (2009) Fire in the Earth system. Science 324:481–484
2. Nemery B, Hoet PH, Nemmar A (2001) The Meuse Valley fog of 1930: an air pollution disaster. Lancet 357:704–708
3. Firket J (1936) Fog along the Meuse Valley. Trans Faraday Soc 32:1192–1197
4. London Ministry of Health (1954) Mortality and morbidity during the London Fog of December 1952. Reports on Public Health and Medical Subjects no 95, London
5. Dooley EE (2002) Fifty years later: clearing the air over the London smog. Environ Health Perspect 110:A748
6. Bown W (1994) Dying from too much dust. New Scientist 12:12–13
7. Pope CA 3rd (1989) Respiratory disease associated with community air pollution and a steel mill, Utah Valley. Am J Public Health 79:623–628
8. Ghio AJ (2004) Biological effects of Utah Valley ambient air particles in humans: a review. J Aerosol Med 17:157–164
9. Daniels MJ, Dominici F, Samet JM, Zeger SL (2000) Estimating particulate matter-mortality dose-response curves and threshold levels: an analysis of daily time-series for the 20 largest US cities. Am J Epidemiol 152:397–406
10. Dockery DW, Pope CA 3rd, Xu X, Spengler JD, Ware JH, Fay ME, Ferris BG Jr, Speizer FE (1993) An association between air pollution and mortality in six U.S. cities. N Engl J Med 329:1753–1759
11. Pope CA 3rd, Burnett RT, Thun MJ, Calle EE, Krewski D, Ito K, Thurston GD (2002) Lung cancer, cardiopulmonary mortality, and long-term exposure to fine particulate air pollution. J Am Med Assoc 287:1132–1141
12. Clancy L, Goodman P, Sinclair H, Dockery DW (2002) Effect of air-pollution control on death rates in Dublin, Ireland: an intervention study. Lancet 360:1210–1214
13. Schins RP, Lightbody JH, Borm PJ, Shi T, Donaldson K, Stone V (2004) Inflammatory effects of coarse and fine particulate matter in relation to chemical and biological constituents. Toxicol Appl Pharmacol 195:1–11
14. Turnbull AB, Harrison RM (2000) Major component contributions to PM10 composition in the UK atmosphere. Atmos Environ 34:3129–3137
15. Lippmann M (2000) Environmental toxicants. Wiley, New York, NY
16. Oberdörster G, Oberdörster E, Oberdörster J (2005) Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ Health Perspect 113:823–839
17. Li N, Sioutas C, Cho A, Schmitz D, Misra C, Sempf J, Wang M, Oberley T, Froines J, Nel A (2003) Ultrafine particulate pollutants induce oxidative stress and mitochondrial damage. Environ Health Perspect 111:455–460
18. Scheepers PT, Bos RP (1992) Combustion of diesel fuel from a toxicological perspective I. Origin of incomplete combustion products. Int Arch Occup Environ Health 64:149–161
19. Saldiva PH, Clarke RW, Coull BA, Stearns RC, Lawrence J, Murthy GG, Diaz E, Koutrakis P, Suh H, Tsuda A, Godleski JJ (2002) Lung inflammation induced by concentrated ambient air particles is related to particle composition. Am J Respir Crit Care Med 165:1610–1617
20. WHO (2006) Air quality guidelines for particulate matter, ozone, nitrogen dioxide and sulfur dioxide—summary of risk assessment. World Health Organization, Geneva, Switzerland. Online available at http://www.who.int/phe/health_ topics/outdoorair_aqg/en/
21. Kreyling WG, Möller W, Semmler-Behnke M, Oberdörster G (2007) Particle dosimetry: deposition and clearance from the respiratory tract and translocation towards extra-pulmonary sites. In: Donaldson K, Borm P (eds) Particle toxicology. CRC, Taylor & Francis, Boca Raton, FL, pp 47–74

22. Wei F, Teng E, Wu G, Hu W, Wilson WE, Chapman RS, Pau PC, Zahang J (1999) Ambient concentrations and elemental compositions of PM10 and PM2.5 in four Chinese cities. Environ Sci Technol 33:4188–4193
23. Pope CA 3rd (2000) Epidemiology of fine particulate air pollution and human health: biologic mechanisms and who's at risk? Environ Health Perspect 108(Suppl 4):713–723
24. Hathout EH, Beeson WL, Nahab F, Rabadi A, Thomas W, Mace JW (2002) Role of exposure to air pollutants in the development of type 1 diabetes before and after 5 year of age. Pediatr Diab 3:184–188
25. Pearson JF, Bachireddy C, Shyamprasad S, Goldfine AB, Brownstein JS (2010) Association between fine particulate matter and diabetes prevalence in the U.S. Diabetes Care 33: 2196–2201
26. Krämer U, Herder C, Sugiri D, Strassburger K, Schikowski T, Ranft U, Rathmann W (2010) Traffic-related air pollution and incident type 2 diabetes: results from the SALIA cohort study. Environ Health Perspect 118:1273–1279
27. UNEP (1994) U.N. Environment Programme and WHO report. Environment 36:4
28. Aust AE, Ball JC, Hu AA, Lighty JS, Smith KR, Straccia AM, Veranth JM, Young WC (2002) Particle characteristics responsible for effects on human lung epithelial cells. Res Rep Health Eff Inst 110:1–65 (discussion 67–76)
29. Ghio AJ, Devlin RB (2001) Inflammatory lung injury after bronchial instillation of air pollution particles. Am J Respir Crit Care Med 164:704–708
30. Molinelli AR, Madden MC, McGee JK, Stonehuerner JG, Ghio AJ (2002) Effect of metal removal on the toxicity of airborne particulate matter from the Utah Valley. Inhal Toxicol 14:1069–1086
31. Donaldson K, Stone V, Borm PJ, Jimenez LA, Gilmour PS, Schins RP, Knaapen AM, Rahman I, Faux SP, Brown DM, MacNee W (2003) Oxidative stress and calcium signaling in the adverse effects of environmental particles (PM10). Free Radic Biol Med 34: 1369–1382
32. Seaton A, MacNee W, Donaldson K, Godden D (1995) Particulate air pollution and acute health effects. Lancet 345:176–178
33. Monn C, Becker S (1999) Cytotoxicity and induction of proinflammatory cytokines from human monocytes exposed to fine (PM2.5) and coarse particles (PM10-2.5) in outdoor and indoor air. Toxicol Appl Pharmacol 155:245–252
34. Bonvallot V, Baeza-Squiban A, Baulig A, Brulant S, Boland S, Muzeau F, Barouki R, Marano F (2001) Organic compounds from diesel exhaust particles elicit a proinflammatory response in human airway epithelial cells and induce cytochrome p450 1A1 expression. Am J Respir Cell Mol Biol 25:515–521
35. Nel AE, Diaz-Sanchez D, Li N (2001) The role of particulate pollutants in pulmonary inflammation and asthma: evidence for the involvement of organic chemicals and oxidative stress. Curr Opin Pulm Med 7:20–26
36. Donaldson K, Tran CL (2002) Inflammation caused by particles and fibers. Inhal Toxicol 14:5–27
37. Lee JT, Son JY, Cho YS (2007) The adverse effects of fine particle air pollution on respiratory function in the elderly. Sci Total Environ 385:28–36
38. Zanobetti A, Schwartz J (2001) Are diabetics more susceptible to the health effects of airborne particles? Am J Respir Crit Care Med 164:831–833
39. Yang IA, Fong KM, Zimmerman PV, Holgate ST, Holloway JW (2008) Genetic susceptibility to the respiratory effects of air pollution. Thorax 63:555–563
40. Nel AE, Diaz-Sanchez D, Ng D, Hiura T, Saxon A (1998) Enhancement of allergic inflammation by the interaction between diesel exhaust particles and the immune system. J Allergy Clin Immunol 102:539–554
41. Samet JM, Dominici F, Curriero FC, Coursac I, Zeger SL (2000) Fine particulate air pollution and mortality in 20 U.S. cities, 1987-1994. N Engl J Med 343:1742–1749

42. MacNee W, Donaldson K (2000) Exacerbations of COPD: environmental mechanisms. Chest 117:390S–397S
43. Sint T, Donohue JF, Ghio AJ (2008) Ambient air pollution particles and the acute exacerbation of chronic obstructive pulmonary disease. Inhal Toxicol 20:25–29
44. Wordley J, Walters S, Ayres JG (1997) Short term variations in hospital admissions and mortality and particulate air pollution. Occup Environ Med 54:108–116
45. Churg A, Brauer M, del Carmen Avila-Casado M, Fortoul TI, Wright JL (2003) Chronic exposure to high levels of particulate air pollution and small airway remodeling. Environ Health Perspect 111:714–718
46. Bonner JC (2007) Lung fibrotic responses to particle exposure. Toxicol Pathol 35:148–153
47. Li MO, Wan YY, Sanjabi S, Robertson AK, Flavell RA (2006) Transforming growth factor-β regulation of immune responses. Annu Rev Immunol 24:99–146
48. Vanhee D, Gosset P, Wallaert B, Voisin C, Tonnel AB (1994) Mechanisms of fibrosis in coal workers' pneumoconiosis. Increased production of platelet-derived growth factor, insulin-like growth factor type I, and transforming growth factor beta and relationship to disease severity. Am J Respir Crit Care Med 150:1049–1055
49. Yamashita N, Sekine K, Miyasaka T, Kawashima R, Nakajima Y, Nakano J, Yamamoto T, Horiuchi T, Hirai K, Ohta K (2001) Platelet-derived growth factor is involved in the augmentation of airway responsiveness through remodeling of airways in diesel exhaust particulate-treated mice. J Allergy Clin Immunol 107:135–142
50. Zhang K, Phan SH (1996) Cytokines and pulmonary fibrosis. Biol Signals 5:232–239
51. Wynn TA (2008) Cellular and molecular mechanisms of fibrosis. J Pathol 214:199–210
52. Hunninghake GW, Garrett KC, Richerson HB, Fantone JC, Ward PA, Rennard SI, Bitterman PB, Crystal RG (1984) Pathogenesis of the granulomatous lung diseases. Am Rev Respir Dis 130:476–496
53. von Klot S, Wolke G, Tuch T, Heinrich J, Dockery DW, Schwartz J, Kreyling WG, Wichmann HE, Peters A (2002) Increased asthma medication use in association with ambient fine and ultrafine particles. Eur Respir J 20:691–702
54. de Haar C, Hassing I, Bol M, Bleumink R, Pieters R (2006) Ultrafine but not fine particulate matter causes airway inflammation and allergic airway sensitization to co-administered antigen in mice. Clin Exp Allergy 36:1469–1479
55. Kleinman MT, Sioutas C, Froines JR, Fanning E, Hamade A, Mendez L, Meacher D, Oldham M (2007) Inhalation of concentrated ambient particulate matter near a heavily trafficked road stimulates antigen-induced airway responses in mice. Inhal Toxicol 19 (Suppl 1):117–126
56. de Haar C, Kool M, Hassing I, Bol M, Lambrecht BN, Pieters R (2008) Lung dendritic cells are stimulated by ultrafine particles and play a key role in particle adjuvant activity. J Allergy Clin Immunol 121:1246–1254
57. Inoue K, Koike E, Takano H, Yanagisawa R, Ichinose T, Yoshikawa T (2009) Effects of diesel exhaust particles on antigen-presenting cells and antigen-specific Th immunity in mice. Exp Biol Med 234:200–209
58. Provoost S, Maes T, Willart MA, Joos GF, Lambrecht BN, Tournoy KG (2010) Diesel exhaust particles stimulate adaptive immunity by acting on pulmonary dendritic cells. J Immunol 184:426–432
59. Laden F, Schwartz J, Speizer FE, Dockery DW (2006) Reduction in fine particulate air pollution and mortality: extended follow-up of the Harvard Six Cities study. Am J Respir Crit Care Med 173:667–672
60. Mauderly JL (2000) Diesel exhaust. In: Lippmann M (ed) Environmental toxicants: human exposure and their health effects. Wiley, New York, NY
61. Schins RP (2002) Mechanisms of genotoxicity of particles and fibers. Inhal Toxicol 14:57–78
62. Azad N, Rojanasakul Y, Vallyathan V (2008) Inflammation and lung cancer: roles of reactive oxygen/nitrogen species. J Toxicol Environ Health B Crit Rev 11:1–15

63. Knaapen AM, Borm PJ, Albrecht C, Schins RP (2004) Inhaled particles and lung cancer part A: mechanisms. Int J Cancer 109:799–809
64. Castranova V, Ma JY, Yang HM, Antonini JM, Butterworth L, Barger MW, Roberts J, Ma JK (2001) Effect of exposure to diesel exhaust particles on the susceptibility of the lung to infection. Environ Health Perspect 109(Suppl 4):609–612
65. Yang HM, Antonini JM, Barger MW, Butterworth L, Roberts BR, Ma JK, Castranova V, Ma JY (2001) Diesel exhaust particles suppress macrophage function and slow the pulmonary clearance of Listeria monocytogenes in rats. Environ Health Perspect 109:515–521
66. Becker S, Soukup JM (1999) Exposure to urban air particulates alters the macrophage-mediated inflammatory response to respiratory viral infection. J Toxicol Environ Health A 57:445–457
67. Mills NL, Donaldson K, Hadoke PW, Boon NA, MacNee W, Cassee FR, Sandström T, Blomberg A, Newby DE (2009) Adverse cardiovascular effects of air pollution. Nat Clin Pract Cardiovasc Med 6:36–44
68. Brook RD (2008) Cardiovascular effects of air pollution. Clin Sci 115:175–187
69. Brook RD, Franklin B, Cascio W, Hong Y, Howard G, Lipsett M, Luepker R, Mittleman M, Samet J, Smith SC Jr, Trager I (2004) Air pollution and cardiovascular disease: a statement for healthcare professionals from the expert panel on population and prevention science of the American Heart Association. Circulation 109:2655–2671
70. Brook RD, Rajagopalan S, Pope CA 3rd, Brook JR, Bhatnagar A, Diez-Roux AV, Holguin F, Hong Y, Luepker RV, Mittleman MA, Peters A, Siscovick D, Smith SC Jr, Whitsel L, Kaufman JD (2010) Particulate matter air pollution and cardiovascular disease: an update to the scientific statement from the American Heart Association. Circulation 121:2331–2378
71. Simkhovich BZ, Kleinman MT, Kloner RA (2009) Particulate air pollution and coronary heart disease. Curr Opin Cardiol 24:604–609
72. O'Toole TE, Hellmann J, Wheat L, Haberzettl P, Lee J, Conklin DJ, Bhatnagar A, Pope CA 3rd (2010) Episodic exposure to fine particulate air pollution decreases circulating levels of endothelial progenitor cells. Circ Res 107:200–203
73. Ruckerl R, Phipps RP, Schneider A, Frampton M, Cyrys J, Oberdörster G, Wichmann HE, Peters A (2007) Ultrafine particles and platelet activation in patients with coronary heart disease—results from a prospective panel study. Part Fibre Toxicol 4:1
74. Rundell KW, Hoffman JR, Caviston R, Bulbulian R, Hollenbach AM (2007) Inhalation of ultrafine and fine particulate matter disrupts systemic vascular function. Inhal Toxicol 19: 133–140
75. Briet M, Collin C, Laurent S, Tan A, Azizi M, Agharazii M, Jeunemaitre X, Alhenc-Gelas F, Boutouyrie P (2007) Endothelial function and chronic exposure to air pollution in normal male subjects. Hypertension 50:970–976
76. Kunzli N, Jerrett M, Mack WJ, Beckerman B, LaBree L, Gilliland F, Thomas D, Peters J, Hodis HN (2005) Ambient air pollution and atherosclerosis in Los Angeles. Environ Health Perspect 113:201–206
77. Mills NL, Törnqvist H, Gonzalez MC, Vink E, Robinson SD, Söderberg S, Boon NA, Donaldson K, Sandström T, Blomberg A, Newby DE (2007) Ischemic and thrombotic effects of dilute diesel-exhaust inhalation in men with coronary heart disease. N Engl J Med 357: 1075–1082
78. Mills NL, Törnqvist H, Robinson SD, Gonzalez M, Darnley K, MacNee W, Boon NA, Donaldson K, Blomberg A, Sandström T, Newby DE (2005) Diesel exhaust inhalation causes vascular dysfunction and impaired endogenous fibrinolysis. Circulation 112:3930–3936
79. Dubowsky SD, Suh H, Schwartz J, Coull BA, Gold DR (2006) Diabetes, obesity, and hypertension may enhance associations between air pollution and markers of systemic inflammation. Environ Health Perspect 114:992–998

80. Schneider A, Neas LM, Graff DW, Herbst MC, Cascio WE, Schmitt MT, Buse JB, Peters A, Devlin RB (2010) Association of cardiac and vascular changes with ambient PM2.5 in diabetic individuals. Part Fibre Toxicol 7:14
81. O'Neill MS, Veves A, Sarnat JA, Zanobetti A, Gold DR, Economides PA, Horton ES, Schwartz J (2007) Air pollution and inflammation in type 2 diabetes: a mechanism for susceptibility. Occup Environ Med 64:373–379
82. Liu L, Ruddy TD, Dalipaj M, Szyszkowicz M, You H, Poon R, Wheeler A, Dales R (2007) Influence of personal exposure to particulate air pollution on cardiovascular physiology and biomarkers of inflammation and oxidative stress in subjects with diabetes. J Occup Environ Med 49:258–265
83. Jacobs L, Emmerechts J, Mathieu C, Hoylaerts MF, Fierens F, Hoet PH, Nemery B, Nawrot TS (2010) Air pollution related prothrombotic changes in persons with diabetes. Environ Health Perspect 118:191–196
84. LeBlanc AJ, Cumpston JL, Chen BT, Frazer D, Castranova V, Nurkiewicz TR (2009) Nanoparticle inhalation impairs endothelium-dependent vasodilation in subepicardial arterioles. J Toxicol Environ Health A 72:1576–1584
85. Nurkiewicz TR, Porter DW, Barger M, Castranova V, Boegehold MA (2004) Particulate matter exposure impairs systemic microvascular endothelium-dependent dilation. Environ Health Perspect 112:1299–1306
86. Nurkiewicz TR, Porter DW, Barger M, Millecchia L, Rao KM, Marvar PJ, Hubbs AF, Castranova V, Boegehold MA (2006) Systemic microvascular dysfunction and inflammation after pulmonary particulate matter exposure. Environ Health Perspect 114:412–419
87. Tamagawa E, Bai N, Morimoto K, Gray C, Mui T, Yatera K, Zhang X, Xing L, Li Y, Laher I, Sin DD, Man SF, van Eeden SF (2008) Particulate matter exposure induces persistent lung inflammation and endothelial dysfunction. Am J Physiol Lung Cell Mol Physiol 295: L79–L85
88. Nemmar A, Hoet PH, Dinsdale D, Vermylen J, Hoylaerts MF, Nemery B (2003) Diesel exhaust particles in lung acutely enhance experimental peripheral thrombosis. Circulation 107:1202–1208
89. Nemmar A, Nemery B, Hoet PH, Vermylen J, Hoylaerts MF (2003) Pulmonary inflammation and thrombogenicity caused by diesel particles in hamsters: role of histamine. Am J Respir Crit Care Med 168:1366–1372
90. Nemmar A, Al-Salam S, Dhanasekaran S, Sudhadevi M, Ali BH (2009) Pulmonary exposure to diesel exhaust particles promotes cerebral microvessel thrombosis: protective effect of a cysteine prodrug L-2-oxothiazolidine-4-carboxylic acid. Toxicology 263:84–92
91. Yokota S, Seki T, Naito Y, Tachibana S, Hirabayashi N, Nakasaka T, Ohara N, Kobayashi H (2008) Tracheal instillation of diesel exhaust particles component causes blood and pulmonary neutrophilia and enhances myocardial oxidative stress in mice. J Toxicol Sci 33: 609–620
92. Cascio WE, Cozzi E, Hazarika S, Devlin RB, Henriksen RA, Lust RM, Van Scott MR, Wingard CJ (2007) Cardiac and vascular changes in mice after exposure to ultrafine particulate matter. Inhal Toxicol 19(Suppl 1):67–73
93. Nurkiewicz TR, Porter DW, Hubbs AF, Cumpston JL, Chen BT, Frazer DG, Castranova V (2008) Nanoparticle inhalation augments particle-dependent systemic microvascular dysfunction. Part Fibre Toxicol 5:1
94. Quan C, Sun Q, Lippmann M, Chen LC (2010) Comparative effects of inhaled diesel exhaust and ambient fine particles on inflammation, atherosclerosis, and vascular dysfunction. Inhal Toxicol 22:738–753
95. Hansen CS, Sheykhzade M, Møller P, Folkmann JK, Amtorp O, Jonassen T, Loft S (2007) Diesel exhaust particles induce endothelial dysfunction in apoE$^{-/-}$ mice. Toxicol Appl Pharmacol 219:24–32

96. Chen LC, Nadziejko C (2005) Effects of subchronic exposures to concentrated ambient particles (CAPs) in mice V. CAPs exacerbate aortic plaque development in hyperlipidemic mice. Inhal Toxicol 17:217–224
97. Sun Q, Wang A, Jin X, Natanzon A, Duquaine D, Brook RD, Aguinaldo JG, Fayad ZA, Fuster V, Lippmann M, Chen LC, Rajagopalan S (2005) Long-term air pollution exposure and acceleration of atherosclerosis and vascular inflammation in an animal model. J Am Med Assoc 294:3003–3010
98. Ying Z, Kampfrath T, Thurston G, Farrar B, Lippmann M, Wang A, Sun Q, Chen LC, Rajagopalan S (2009) Ambient particulates alter vascular function through induction of reactive oxygen and nitrogen species. Toxicol Sci 111:80–88
99. Goto Y, Hogg JC, Shih CH, Ishii H, Vincent R, van Eeden SF (2004) Exposure to ambient particles accelerates monocyte release from bone marrow in atherosclerotic rabbits. Am J Physiol Lung Cell Mol Physiol 287:L79–L85
100. Yatera K, Hsieh J, Hogg JC, Tranfield E, Suzuki H, Shih CH, Behzad AR, Vincent R, van Eeden SF (2008) Particulate matter air pollution exposure promotes recruitment of monocytes into atherosclerotic plaques. Am J Physiol Heart Circ Physiol 294:H944–H953
101. Lei YC, Chen MC, Chan CC, Wang PY, Lee CT, Cheng TJ (2004) Effects of concentrated ambient particles on airway responsiveness and pulmonary inflammation in pulmonary hypertensive rats. Inhal Toxicol 16:785–792
102. Kodavanti UP, Schladweiler MC, Ledbetter AD, Hauser R, Christiani DC, McGee J, Richards JR, Costa DL (2002) Temporal association between pulmonary and systemic effects of particulate matter in healthy and cardiovascular compromised rats. J Toxicol Environ Health A 65:1545–1569
103. Ulrich MM, Alink GM, Kumarathasan P, Vincent R, Boere AJ, Cassee FR (2002) Health effects and time course of particulate matter on the cardiopulmonary system in rats with lung inflammation. J Toxicol Environ Health A 65:1571–1595
104. Nemmar A, Vanbilloen H, Hoylaerts MF, Hoet PH, Verbruggen A, Nemery B (2001) Passage of intratracheally instilled ultrafine particles from the lung into the systemic circulation in hamster. Am J Respir Crit Care Med 164:1665–1668
105. Nemmar A, Hoet PH, Vanquickenborne B, Dinsdale D, Thomeer M, Hoylaerts MF, Vanbilloen H, Mortelmans L, Nemery B (2002) Passage of inhaled particles into the blood circulation in humans. Circulation 105:411–414
106. Kreyling WG, Semmler-Behnke M, Seitz J, Scymczak W, Wenk A, Mayer P, Takenaka S, Oberdörster G (2009) Size dependence of the translocation of inhaled iridium and carbon nanoparticle aggregates from the lung of rats to the blood and secondary target organs. Inhal Toxicol 21(Suppl 1):55–60
107. Semmler-Behnke M, Kreyling WG, Lipka J, Fertsch S, Wenk A, Takenaka S, Schmid G, Brandau W (2008) Biodistribution of 1.4- and 18-nm gold particles in rats. Small 4: 2108–2111
108. Takenaka S, Karg E, Kreyling WG, Lentner B, Möller W, Behnke-Semmler M, Jennen L, Walch A, Michalke B, Schramel P, Heyder J, Schulz H (2006) Distribution pattern of inhaled ultrafine gold particles in the rat lung. Inhal Toxicol 18:733–740
109. Mills NL, Amin N, Robinson SD, Anand A, Davies J, Patel D, de la Fuente JM, Cassee FR, Boon NA, Macnee W, Millar AM, Donaldson K, Newby DE (2006) Do inhaled carbon nanoparticles translocate directly into the circulation in humans? Am J Respir Crit Care Med 173:426–431
110. Möller W, Felten K, Sommerer K, Scheuch G, Meyer G, Meyer P, Haussinger K, Kreyling WG (2008) Deposition, retention, and translocation of ultrafine particles from the central airways and lung periphery. Am J Respir Crit Care Med 177:426–432
111. Wiebert P, Sanchez-Crespo A, Seitz J, Falk R, Philipson K, Kreyling WG, Möller W, Sommerer K, Larsson S, Svartengren M (2006) Negligible clearance of ultrafine particles retained in healthy and affected human lungs. Eur Respir J 28:286–290

112. Donaldson K, Tran L, Jimenez LA, Duffin R, Newby DE, Mills N, MacNee W, Stone V (2005) Combustion-derived nanoparticles: a review of their toxicology following inhalation exposure. Part Fibre Toxicol 2:10
113. Clayton TC, Thompson M, Meade TW (2008) Recent respiratory infection and risk of cardiovascular disease: case-control study through a general practice database. Eur Heart J 29:96–103
114. Ghio AJ, Kim C, Devlin RB (2000) Concentrated ambient air particles induce mild pulmonary inflammation in healthy human volunteers. Am J Respir Crit Care Med 162:981–988
115. Gong H Jr, Linn WS, Sioutas C, Terrell SL, Clark KW, Anderson KR, Terrell LL (2003) Controlled exposures of healthy and asthmatic volunteers to concentrated ambient fine particles in Los Angeles. Inhal Toxicol 15:305–325
116. Rivero DH, Soares SR, Lorenzi-Filho G, Saiki M, Godleski JJ, Antonangelo L, Dolhnikoff M, Saldiva PH (2005) Acute cardiopulmonary alterations induced by fine particulate matter of Sao Paulo, Brazil. Toxicol Sci 85:898–905
117. Rückerl R, Ibald-Mulli A, Koenig W, Schneider A, Woelke G, Cyrys J, Heinrich J, Marder V, Frampton M, Wichmann HE, Peters A (2006) Air pollution and markers of inflammation and coagulation in patients with coronary heart disease. Am J Respir Crit Care Med 173:432–441
118. Schwartz J (2001) Air pollution and blood markers of cardiovascular risk. Environ Health Perspect 109(Suppl 3):405–409
119. Oikonen M, Laaksonen M, Laippala P, Oksaranta O, Lilius EM, Lindgren S, Rantio-Lehtimaki A, Anttinen A, Koski K, Eralinna JP (2003) Ambient air quality and occurrence of multiple sclerosis relapse. Neuroepidemiology 22:95–99
120. Calderon-Garciduenas L, Reed W, Maronpot RR, Henriquez-Roldan C, Delgado-Chavez R, Calderon-Garciduenas A, Dragustinovis I, Franco-Lira M, Aragon-Flores M, Solt AC, Altenburg M, Torres-Jardon R, Swenberg JA (2004) Brain inflammation and Alzheimer's-like pathology in individuals exposed to severe air pollution. Toxicol Pathol 32:650–658
121. Oberdörster G, Sharp Z, Atudorei V, Elder A, Gelein R, Kreyling W, Cox C (2004) Translocation of inhaled ultrafine particles to the brain. Inhal Toxicol 16:437–445
122. Elder A, Gelein R, Silva V, Feikert T, Opanashuk L, Carter J, Potter R, Maynard A, Ito Y, Finkelstein J, Oberdörster G (2006) Translocation of inhaled ultrafine manganese oxide particles to the central nervous system. Environ Health Perspect 114:1172–1178
123. Cruts B, van Etten L, Törnqvist H, Blomberg A, Sandström T, Mills NL, Borm PJ (2008) Exposure to diesel exhaust induces changes in EEG in human volunteers. Part Fibre Toxicol 5:4
124. Ranft U, Schikowski T, Sugiri D, Krutmann J, Kramer U (2009) Long-term exposure to traffic-related particulate matter impairs cognitive function in the elderly. Environ Res 109: 1004–1011
125. Suglia SF, Gryparis A, Wright RO, Schwartz J, Wright RJ (2008) Association of black carbon with cognition among children in a prospective birth cohort study. Am J Epidemiol 167:280–286
126. Calderon-Garciduenas L, Mora-Tiscareno A, Ontiveros E, Gomez-Garza G, Barragan-Mejia G, Broadway J, Chapman S, Valencia-Salazar G, Jewells V, Maronpot RR, Henriquez-Roldan C, Perez-Guille B, Torres-Jardon R, Herrit L, Brooks D, Osnaya-Brizuela N, Monroy ME, Gonzalez-Maciel A, Reynoso-Robles R, Villarreal-Calderon R, Solt AC, Engle RW (2008) Air pollution, cognitive deficits and brain abnormalities: a pilot study with children and dogs. Brain Cogn 68:117–127
127. Campbell A, Oldham M, Becaria A, Bondy SC, Meacher D, Sioutas C, Misra C, Mendez LB, Kleinman M (2005) Particulate matter in polluted air may increase biomarkers of inflammation in mouse brain. Neurotoxicology 26:133–140
128. Kleinman MT, Araujo JA, Nel A, Sioutas C, Campbell A, Cong PQ, Li H, Bondy SC (2008) Inhaled ultrafine particulate matter affects CNS inflammatory processes and may act via MAP kinase signaling pathways. Toxicol Lett 178:127–130
129. Veronesi B, Makwana O, Pooler M, Chen LC (2005) Effects of subchronic exposures to concentrated ambient particles. VII. Degeneration of dopaminergic neurons in Apo $E^{-/-}$ mice. Inhal Toxicol 17:235–241

130. Sirivelu MP, MohanKumar SM, Wagner JG, Harkema JR, MohanKumar PS (2006) Activation of the stress axis and neurochemical alterations in specific brain areas by concentrated ambient particle exposure with concomitant allergic airway disease. Environ Health Perspect 114:870–874
131. Gerlofs-Nijland ME, van Berlo D, Cassee FR, Schins RP, Wang K, Campbell A (2010) Effect of prolonged exposure to diesel engine exhaust on proinflammatory markers in different regions of the rat brain. Part Fibre Toxicol 7:12
132. van Berlo D, Albrecht C, Knaapen AM, Cassee FR, Gerlofs-Nijland ME, Kooter IM, Palomero-Gallagher N, Bidmon HJ, van Schooten FJ, Krutmann J, Schins RP (2010) Comparative evaluation of the effects of short-term inhalation exposure to diesel engine exhaust on rat lung and brain. Arch Toxicol 84:553–562
133. Suzuki T, Oshio S, Iwata M, Saburi H, Odagiri T, Udagawa T, Sugawara I, Umezawa M, Takeda K (2010) *In utero* exposure to a low concentration of diesel exhaust affects spontaneous locomotor activity and monoaminergic system in male mice. Part Fibre Toxicol 7:7
134. Qin L, Wu X, Block ML, Liu Y, Breese GR, Hong JS, Knapp DJ, Crews FT (2007) Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. Glia 55:453–462
135. Rivest S (2001) How circulating cytokines trigger the neural circuits that control the hypothalamic-pituitary-adrenal axis. Psychoneuroendocrinology 26:761–788
136. Lim HB, Ichinose T, Miyabara Y, Takano H, Kumagai Y, Shimojyo N, Devalia JL, Sagai M (1998) Involvement of superoxide and nitric oxide on airway inflammation and hyperresponsiveness induced by diesel exhaust particles in mice. Free Radic Biol Med 25:635–644
137. Gurgueira SA, Lawrence J, Coull B, Murthy GG, Gonzalez-Flecha B (2002) Rapid increases in the steady-state concentration of reactive oxygen species in the lungs and heart after particulate air pollution inhalation. Environ Health Perspect 110:749–755
138. Li N, Xia T, Nel AE (2008) The role of oxidative stress in ambient particulate matter-induced lung diseases and its implications in the toxicity of engineered nanoparticles. Free Radic Biol Med 44:1689–1699
139. Xiao GG, Wang M, Li N, Loo JA, Nel AE (2003) Use of proteomics to demonstrate a hierarchical oxidative stress response to diesel exhaust particle chemicals in a macrophage cell line. J Biol Chem 278:50781–50790
140. Donaldson K, MacNee W (1998) The mechanism of lung injury by PM10. In: Hester RE, Harrison RM (eds) Air pollution and health. The Royal Society of Chemistry, pp 21–32. Online available at http://www.knovel.com/web/portal/browse/display?_EXT_KNOVEL_-DISPLAY_ bookid=644
141. Sies H (1985) Oxidative stress: introductory remarks. In: Sies H (ed) Oxidative stress. Academic, London, pp 1–8
142. Knaapen AM, Shi T, Borm PJ, Schins RP (2002) Soluble metals as well as the insoluble particle fraction are involved in cellular DNA damage induced by particulate matter. Mol Cell Biochem 234–235:317–326
143. Shi T, Knaapen AM, Begerow J, Birmili W, Borm PJ, Schins RP (2003) Temporal variation of hydroxyl radical generation and 8-hydroxy-2′-deoxyguanosine formation by coarse and fine particulate matter. Occup Environ Med 60:315–321
144. Shi T, Duffin R, Borm PJ, Li H, Weishaupt C, Schins RP (2006) Hydroxyl-radical-dependent DNA damage by ambient particulate matter from contrasting sampling locations. Environ Res 101:18–24
145. Schaumann F, Borm PJ, Herbrich A, Knoch J, Pitz M, Schins RP, Luettig B, Hohlfeld JM, Heinrich J, Krug N (2004) Metal-rich ambient particles (particulate matter 2.5) cause airway inflammation in healthy subjects. Am J Respir Crit Care Med 170:898–903
146. Baulig A, Poirault JJ, Ausset P, Schins R, Shi T, Baralle D, Dorlhene P, Meyer M, Lefevre R, Baeza-Squiban A, Marano F (2004) Physicochemical characteristics and biological activities of seasonal atmospheric particulate matter sampling in two locations of Paris. Environ Sci Technol 38:5985–5992
147. Brown DM, Wilson MR, MacNee W, Stone V, Donaldson K (2001) Size-dependent proinflammatory effects of ultrafine polystyrene particles: a role for surface area and oxidative stress in the enhanced activity of ultrafines. Toxicol Appl Pharmacol 175:191–199

148. Wessels A, Van Berlo D, Boots AW, Gerloff K, Scherbart A, Cassee FR, Gerlofs-Nijland ME, Van Schooten FJ, Albrecht C, Schins RP (2011) Oxidative stress and DNA damage responses in rat and mouse lung to inhaled carbon nanoparticles. Nanotoxicology 5:66–78
149. Donaldson K, Brown DM, Mitchell C, Dineva M, Beswick PH, Gilmour P, MacNee W (1997) Free radical activity of PM10: iron-mediated generation of hydroxyl radicals. Environ Health Perspect 105(Suppl 5):1285–1289
150. Squadrito GL, Cueto R, Dellinger B, Pryor WA (2001) Quinoid redox cycling as a mechanism for sustained free radical generation by inhaled airborne particulate matter. Free Radic Biol Med 31:1132–1138
151. Baulig A, Sourdeval M, Meyer M, Marano F, Baeza-Squiban A (2003) Biological effects of atmospheric particles on human bronchial epithelial cells. Comparison with diesel exhaust particles. Toxicol In Vitro 17:567–573
152. Ades EW, Candal FJ, Swerlick RA, George VG, Summers S, Bosse DC, Lawley TJ (1992) HMEC-1: establishment of an immortalized human microvascular endothelial cell line. J Invest Dermatol 99:683–690
153. Penning TM, Burczynski ME, Hung CF, McCoull KD, Palackal NT, Tsuruda LS (1999) Dihydrodiol dehydrogenases and polycyclic aromatic hydrocarbon activation: generation of reactive and redox active *o*-quinones. Chem Res Toxicol 12:1–18
154. DosReis GA, Borges VM (2003) Role of Fas-ligand induced apoptosis in pulmonary inflammation and injury. Curr Drug Targets Inflamm Allergy 2:161–167
155. Shepherd VL (1986) The role of the respiratory burst of phagocytes in host defense. Semin Respir Infect 1:99–106
156. Segal AW (2005) How neutrophils kill microbes. Ann Rev Immunol 23:197–223
157. Emmendoerffer A, Hecht M, Boeker T, Mueller M, Heinrich U (2000) Role of inflammation in chemical-induced lung cancer. Toxicol Lett 112–113:185–191
158. Hancock JT, Desikan R, Neill SJ (2001) Role of reactive oxygen species in cell signalling pathways. Biochem Soc Trans 29:345–350
159. Shacter E, Weitzman SA (2002) Chronic inflammation and cancer. Oncology 16(217–226):229 (discussion 230–212)
160. Knaapen AM, Gungor N, Schins RP, Borm PJ, Van Schooten FJ (2006) Neutrophils and respiratory tract DNA damage and mutagenesis: a review. Mutagenesis 21:225–236
161. Sips HJ, Hamers MN (1981) Mechanism of the bactericidal action of myeloperoxidase: increased permeability of the *Escherichia coli* cell envelope. Infect Immun 31:11–16
162. Guo FH, De Raeve HR, Rice TW, Stuehr DJ, Thunnissen FB, Erzurum SC (1995) Continuous nitric oxide synthesis by inducible nitric oxide synthase in normal human airway epithelium *in vivo*. Proc Natl Acad Sci USA 92:7809–7813
163. Robbins RA, Barnes PJ, Springall DR, Warren JB, Kwon OJ, Buttery LD, Wilson AJ, Geller DA, Polak JM (1994) Expression of inducible nitric oxide in human lung epithelial cells. Biochem Biophys Res Commun 203:209–218
164. Taniyama Y, Griendling KK (2003) Reactive oxygen species in the vasculature: molecular and cellular mechanisms. Hypertension 42:1075–1081
165. Wu LL, Chiou CC, Chang PY, Wu JT (2004) Urinary 8-OHdG: a marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics. Clin Chim Acta 339:1–9
166. Marnett LJ (2000) Oxyradicals and DNA damage. Carcinogenesis 21:361–370
167. Schins RP, Knaapen AM (2007) Genotoxicity of poorly soluble particles. Inhal Toxicol 19 (Suppl 1):189–198
168. Schraufstatter I, Hyslop PA, Jackson JH, Cochrane CG (1988) Oxidant-induced DNA damage of target cells. J Clin Invest 82:1040–1050
169. Chong YC, Heppner GH, Paul LA, Fulton AM (1989) Macrophage-mediated induction of DNA strand breaks in target tumor cells. Cancer Res 49:6652–6657
170. Driscoll KE, Deyo LC, Carter JM, Howard BW, Hassenbein DG, Bertram TA (1997) Effects of particle exposure and particle-elicited inflammatory cells on mutation in rat alveolar epithelial cells. Carcinogenesis 18:423–430

171. Weitzman SA, Weitberg AB, Clark EP, Stossel TP (1985) Phagocytes as carcinogens: malignant transformation produced by human neutrophils. Science 227:1231–1233
172. Shacter E, Beecham EJ, Covey JM, Kohn KW, Potter M (1988) Activated neutrophils induce prolonged DNA damage in neighboring cells. Carcinogenesis 9:2297–2304
173. Knaapen AM, Schins RP, Borm PJ, van Schooten FJ (2005) Nitrite enhances neutrophil-induced DNA strand breakage in pulmonary epithelial cells by inhibition of myeloperoxidase. Carcinogenesis 26:1642–1648
174. Knaapen AM, Seiler F, Schilderman PA, Nehls P, Bruch J, Schins RP, Borm PJ (1999) Neutrophils cause oxidative DNA damage in alveolar epithelial cells. Free Radic Biol Med 27:234–240
175. Driscoll KE, Hassenbein DG, Howard BW, Isfort RJ, Cody D, Tindal MH, Suchanek M, Carter JM (1995) Cloning, expression, and functional characterization of rat MIP-2: a neutrophil chemoattractant and epithelial cell mitogen. J Leukoc Biol 58:359–364
176. Murphy PM (1997) Neutrophil receptors for interleukin-8 and related CXC chemokines. Semin Hematol 34:311–318
177. Tracey KJ, Cerami A (1993) Tumor necrosis factor, other cytokines and disease. Annu Rev Cell Biol 9:317–343
178. Vassalli P (1992) The pathophysiology of tumor necrosis factors. Annu Rev Immunol 10: 411–452
179. Chung KF (2001) Cytokines in chronic obstructive pulmonary disease. Eur Respir J Suppl 34:50s–59s
180. Christman JW, Sadikot RT, Blackwell TS (2000) The role of nuclear factor-κB in pulmonary diseases. Chest 117:1482–1487
181. Lee JI, Burckart GJ (1998) Nuclear factor κB: important transcription factor and therapeutic target. J Clin Pharmacol 38:981–993
182. Kleinert H, Euchenhofer C, Ihrig-Biedert I, Forstermann U (1996) In murine 3T3 fibroblasts, different second messenger pathways resulting in the induction of NO synthase II (iNOS) converge in the activation of transcription factor NF-κB. J Biol Chem 271:6039–6044
183. Schmedtje JF Jr, Ji YS, Liu WL, DuBois RN, Runge MS (1997) Hypoxia induces cyclooxygenase-2 via the NF-κB p65 transcription factor in human vascular endothelial cells. J Biol Chem 272:601–608
184. Collart MA, Baeuerle P, Vassalli P (1990) Regulation of tumor necrosis factor α transcription in macrophages: involvement of four κB-like motifs and of constitutive and inducible forms of NF-κB. Mol Cell Biol 10:1498–1506
185. Shakhov AN, Collart MA, Vassalli P, Nedospasov SA, Jongeneel CV (1990) Kappa B-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor α gene in primary macrophages. J Exp Med 171:35–47
186. Auron PE, Webb AC (1994) Interleukin-1: a gene expression system regulated at multiple levels. Eur Cytokine Netw 5:573–592
187. Sanceau J, Kaisho T, Hirano T, Wietzerbin J (1995) Triggering of the human interleukin-6 gene by interferon-γ and tumor necrosis factor-α in monocytic cells involves cooperation between interferon regulatory factor-1, NFκB, and Sp1 transcription factors. J Biol Chem 270:27920–27931
188. Harant H, de Martin R, Andrew PJ, Foglar E, Dittrich C, Lindley IJ (1996) Synergistic activation of interleukin-8 gene transcription by *all-trans*-retinoic acid and tumor necrosis factor-α involves the transcription factor NFκB. J Biol Chem 271:26954–26961
189. Roebuck KA, Rahman A, Lakshminarayanan V, Janakidevi K, Malik AB (1995) H_2O_2 and tumor necrosis factor-α activate intercellular adhesion molecule 1 (ICAM-1) gene transcription through distinct cis-regulatory elements within the ICAM-1 promoter. J Biol Chem 270:18966–18974
190. Dunn SM, Coles LS, Lang RK, Gerondakis S, Vadas MA, Shannon MF (1994) Requirement for nuclear factor (NF)-κB p65 and NF-interleukin-6 binding elements in the tumor necrosis factor response region of the granulocyte colony-stimulating factor promoter. Blood 83: 2469–2479

191. Israel A, Le Bail O, Hatat D, Piette J, Kieran M, Logeat F, Wallach D, Fellous M, Kourilsky P (1989) TNF stimulates expression of mouse MHC class I genes by inducing an NF κB-like enhancer binding activity which displaces constitutive factors. EMBO J 8:3793–3800
192. Osborn L, Kunkel S, Nabel GJ (1989) Tumor necrosis factor α and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor κB. Proc Natl Acad Sci USA 86:2336–2340
193. Ishii H, Fujii T, Hogg JC, Hayashi S, Mukae H, Vincent R, van Eeden SF (2004) Contribution of IL-1 β and TNF-α to the initiation of the peripheral lung response to atmospheric particulates (PM10). Am J Physiol Lung Cell Mol Physiol 287:L176–L183
194. Jimenez LA, Drost EM, Gilmour PS, Rahman I, Antonicelli F, Ritchie H, MacNee W, Donaldson K (2002) PM(10)-exposed macrophages stimulate a proinflammatory response in lung epithelial cells via TNF-α. Am J Physiol Lung Cell Mol Physiol 282:L237–L248
195. Janssen-Heininger YM, Macara I, Mossman BT (1999) Cooperativity between oxidants and tumor necrosis factor in the activation of nuclear factor (NF)-κB: requirement of Ras/mitogen-activated protein kinases in the activation of NF-κB by oxidants. Am J Respir Cell Mol Biol 20:942–952
196. Crapo JD, Barry BE, Gehr P, Bachofen M, Weibel ER (1982) Cell number and cell characteristics of the normal human lung. Am Rev Respir Dis 126:332–337
197. Laing S, Wang G, Briazova T, Zhang C, Wang A, Zheng Z, Gow A, Chen AF, Rajagopalan S, Chen LC, Sun Q, Zhang K (2010) Airborne particulate matter selectively activates endoplasmic reticulum stress response in the lung and liver tissues. Am J Physiol Cell Physiol 299:C736–C749
198. Lundqvist M, Stigler J, Elia G, Lynch I, Cedervall T, Dawson KA (2008) Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts. Proc Natl Acad Sci USA 105:14265–14270
199. Kilroe-Smith TA, Webster I, Van Drimmelen M, Marasas L (1973) An insoluble fibrogenic factor in macrophages from guinea pigs exposed to silica. Environ Res 6:298–305
200. Rabinovitch M, De Stefano MJ (1973) Particle recognition by cultivated macrophages. J Immunol 110:695–701
201. Bowden DH (1976) The pulmonary macrophage. Environ Health Perspect 16:55–60
202. Bowden DH (1984) The alveolar macrophage. Environ Health Perspect 55:327–341
203. Lindbom J, Gustafsson M, Blomqvist G, Dahl A, Gudmundsson A, Swietlicki E, Ljungman AG (2006) Exposure to wear particles generated from studded tires and pavement induces inflammatory cytokine release from human macrophages. Chem Res Toxicol 19:521–530
204. den Hartigh LJ, Lame MW, Ham W, Kleeman MJ, Tablin F, Wilson DW (2010) Endotoxin and polycyclic aromatic hydrocarbons in ambient fine particulate matter from Fresno, California initiate human monocyte inflammatory responses mediated by reactive oxygen species. Toxicol In Vitro 24:1993–2002
205. Sakamoto N, Hayashi S, Mukae H, Vincent R, Hogg JC, van Eeden SF (2009) Effect of atorvastatin on PM10-induced cytokine production by human alveolar macrophages and bronchial epithelial cells. Int J Toxicol 28:17–23
206. Kocbach A, Namork E, Schwarze PE (2008) Pro-inflammatory potential of wood smoke and traffic-derived particles in a monocytic cell line. Toxicology 247:123–132
207. Brown DM, Donaldson K, Stone V (2004) Effects of PM10 in human peripheral blood monocytes and J774 macrophages. Respir Res 5:29
208. Brown DM, Hutchison L, Donaldson K, Stone V (2007) The effects of PM10 particles and oxidative stress on macrophages and lung epithelial cells: modulating effects of calcium-signaling antagonists. Am J Physiol Lung Cell Mol Physiol 292:L1444–L1451
209. Alexis NE, Lay JC, Zeman K, Bennett WE, Peden DB, Soukup JM, Devlin RB, Becker S (2006) Biological material on inhaled coarse fraction particulate matter activates airway phagocytes *in vivo* in healthy volunteers. J Allergy Clin Immunol 117:1396–1403
210. Hofer TP, Bitterle E, Beck-Speier I, Maier KL, Frankenberger M, Heyder J, Ziegler-Heitbrock L (2004) Diesel exhaust particles increase LPS-stimulated COX-2 expression and PGE_2 production in human monocytes. J Leukoc Biol 75:856–864

211. Vogel CF, Sciullo E, Wong P, Kuzmicky P, Kado N, Matsumura F (2005) Induction of proinflammatory cytokines and C-reactive protein in human macrophage cell line U937 exposed to air pollution particulates. Environ Health Perspect 113:1536–1541
212. Beck-Speier I, Dayal N, Karg E, Maier KL, Schumann G, Schulz H, Semmler M, Takenaka S, Stettmaier K, Bors W, Ghio A, Samet JM, Heyder J (2005) Oxidative stress and lipid mediators induced in alveolar macrophages by ultrafine particles. Free Radic Biol Med 38:1080–1092
213. Mondal K, Stephen Haskill J, Becker S (2000) Adhesion and pollution particle-induced oxidant generation is neither necessary nor sufficient for cytokine induction in human alveolar macrophages. Am J Respir Cell Mol Biol 22:200–208
214. Pozzi R, De Berardis B, Paoletti L, Guastadisegni C (2003) Inflammatory mediators induced by coarse (PM2.5-10) and fine (PM2.5) urban air particles in RAW 264.7 cells. Toxicology 183:243–254
215. Pozzi R, De Berardis B, Paoletti L, Guastadisegni C (2005) Winter urban air particles from Rome (Italy): effects on the monocytic-macrophagic RAW 264.7 cell line. Environ Res 99:344–354
216. Dick CA, Brown DM, Donaldson K, Stone V (2003) The role of free radicals in the toxic and inflammatory effects of four different ultrafine particle types. Inhal Toxicol 15:39–52
217. Soukup JM, Becker S (2001) Human alveolar macrophage responses to air pollution particulates are associated with insoluble components of coarse material, including particulate endotoxin. Toxicol Appl Pharmacol 171:20–26
218. Becker S, Mundandhara S, Devlin RB, Madden M (2005) Regulation of cytokine production in human alveolar macrophages and airway epithelial cells in response to ambient air pollution particles: further mechanistic studies. Toxicol Appl Pharmacol 207:269–275
219. Jalava PI, Salonen RO, Pennanen AS, Sillanpää M, Hälinen AI, Happo MS, Hillamo R, Brunekreef B, Katsouyanni K, Sunyer J, Hirvonen MR (2007) Heterogeneities in inflammatory and cytotoxic responses of RAW 264.7 macrophage cell line to urban air coarse, fine, and ultrafine particles from six European sampling campaigns. Inhal Toxicol 19:213–225
220. Happo MS, Salonen RO, Hälinen AI, Jalava PI, Pennanen AS, Dormans JA, Gerlofs-Nijland ME, Cassee FR, Kosma VM, Sillanpää M, Hillamo R, Hirvonen MR (2010) Inflammation and tissue damage in mouse lung by single and repeated dosing of urban air coarse and fine particles collected from six European cities. Inhal Toxicol 22:402–416
221. Ishii H, Hayashi S, Hogg JC, Fujii T, Goto Y, Sakamoto N, Mukae H, Vincent R, van Eeden SF (2005) Alveolar macrophage-epithelial cell interaction following exposure to atmospheric particles induces the release of mediators involved in monocyte mobilization and recruitment. Respir Res 6:87
222. Kocbach A, Herseth JI, Lag M, Refsnes M, Schwarze PE (2008) Particles from wood smoke and traffic induce differential pro-inflammatory response patterns in co-cultures. Toxicol Appl Pharmacol 232:317–326
223. Becker S, Soukup J (2003) Coarse(PM(2.5-10)), fine(PM(2.5)), and ultrafine air pollution particles induce/increase immune costimulatory receptors on human blood-derived monocytes but not on alveolar macrophages. J Toxicol Environ Health A 66:847–859
224. Müller L, Riediker M, Wick P, Mohr M, Gehr P, Rothen-Rutishauser B (2010) Oxidative stress and inflammation response after nanoparticle exposure: differences between human lung cell monocultures and an advanced three-dimensional model of the human epithelial airways. J R Soc Interface 7(Suppl 1):S27–S40
225. Ohyama M, Otake T, Adachi S, Kobayashi T, Morinaga K (2007) A comparison of the production of reactive oxygen species by suspended particulate matter and diesel exhaust particles with macrophages. Inhal Toxicol 19(Suppl 1):157–160
226. Zhang Y, Schauer JJ, Shafer MM, Hannigan MP, Dutton SJ (2008) Source apportionment of *in vitro* reactive oxygen species bioassay activity from atmospheric particulate matter. Environ Sci Technol 42:7502–7509

227. Huang YC, Soukup J, Harder S, Becker S (2003) Mitochondrial oxidant production by a pollutant dust and NO-mediated apoptosis in human alveolar macrophage. Am J Physiol Cell Physiol 284:C24–C32
228. Becker S, Soukup JM, Gallagher JE (2002) Differential particulate air pollution induced oxidant stress in human granulocytes, monocytes and alveolar macrophages. Toxicol In Vitro 16:209–218
229. Aam BB, Fonnum F (2007) Carbon black particles increase reactive oxygen species formation in rat alveolar macrophages *in vitro*. Arch Toxicol 81:441–446
230. Chauhan V, Breznan D, Goegan P, Nadeau D, Karthikeyan S, Brook JR, Vincent R (2004) Effects of ambient air particles on nitric oxide production in macrophage cell lines. Cell Biol Toxicol 20:221–239
231. Lindbom J, Gustafsson M, Blomqvist G, Dahl A, Gudmundsson A, Swietlicki E, Ljungman AG (2007) Wear particles generated from studded tires and pavement induces inflammatory reactions in mouse macrophage cells. Chem Res Toxicol 20:937–946
232. Salonen RO, Halinen AI, Pennanen AS, Hirvonen MR, Sillanpää M, Hillamo R, Shi T, Borm P, Sandell E, Koskentalo T, Aarnio P (2004) Chemical and *in vitro* toxicologic characterization of wintertime and springtime urban-air particles with an aerodynamic diameter below 10 microm in Helsinki. Scand J Work Environ Health 30(Suppl 2):80–90
233. Diociaiuti M, Balduzzi M, De Berardis B, Cattani G, Stacchini G, Ziemacki G, Marconi A, Paoletti L (2001) The two PM(2.5) (fine) and PM(2.5-10) (coarse) fractions: evidence of different biological activity. Environ Res 86:254–262
234. Becker S, Soukup JM (1998) Decreased CD11b expression, phagocytosis, and oxidative burst in urban particulate pollution-exposed human monocytes and alveolar macrophages. J Toxicol Environ Health A 55:455–477
235. Kleinman MT, Sioutas C, Chang MC, Boere AJ, Cassee FR (2003) Ambient fine and coarse particle suppression of alveolar macrophage functions. Toxicol Lett 137:151–158
236. Yang HM, Barger MW, Castranova V, Ma JK, Yang JJ, Ma JY (1999) Effects of diesel exhaust particles (DEP), carbon black, and silica on macrophage responses to lipopolysaccharide: evidence of DEP suppression of macrophage activity. J Toxicol Environ Health A 58:261–278
237. Amakawa K, Terashima T, Matsuzaki T, Matsumaru A, Sagai M, Yamaguchi K (2003) Suppressive effects of diesel exhaust particles on cytokine release from human and murine alveolar macrophages. Exp Lung Res 29:149–164
238. Möller W, Brown DM, Kreyling WG, Stone V (2005) Ultrafine particles cause cytoskeletal dysfunctions in macrophages: role of intracellular calcium. Part Fibre Toxicol 2:7
239. Huang YC, Li Z, Harder SD, Soukup JM (2004) Apoptotic and inflammatory effects induced by different particles in human alveolar macrophages. Inhal Toxicol 16:863–878
240. Lentsch AB, Shanley TP, Sarma V, Ward PA (1997) *In vivo* suppression of NF-κB and preservation of IκBα by interleukin-10 and interleukin-13. J Clin Invest 100:2443–2448
241. Monteiller C, Tran L, MacNee W, Faux S, Jones A, Miller B, Donaldson K (2007) The pro-inflammatory effects of low-toxicity low-solubility particles, nanoparticles and fine particles, on epithelial cells *in vitro*: the role of surface area. Occup Environ Med 64:609–615
242. Baeza-Squiban A, Bonvallot V, Boland S, Marano F (1999) Diesel exhaust particles increase NF-κB DNA binding activity and *c-FOS* proto-oncogene expression in human bronchial epithelial cells. Toxicol In Vitro 13:817–822
243. Boland S, Bonvallot V, Fournier T, Baeza-Squiban A, Aubier M, Marano F (2000) Mechanisms of GM-CSF increase by diesel exhaust particles in human airway epithelial cells. Am J Physiol Lung Cell Mol Physiol 278:L25–L32
244. Hashimoto S, Gon Y, Takeshita I, Matsumoto K, Jibiki I, Takizawa H, Kudoh S, Horie T (2000) Diesel exhaust particles activate p38 MAP kinase to produce interleukin 8 and RANTES by human bronchial epithelial cells and *N*-acetylcysteine attenuates p38 MAP kinase activation. Am J Respir Crit Care Med 161:280–285

245. Knaapen AM, Schins RP, Steinfartz Y, Höhr D, Dunemann L, Borm PJ (2000) Ambient particulate matter induces oxidative DNA damage in lung epithelial cells. Inhal Toxicol 12:125–132
246. Don Porto Carero A, Hoet PH, Verschaeve L, Schoeters G, Nemery B (2001) Genotoxic effects of carbon black particles, diesel exhaust particles, and urban air particulates and their extracts on a human alveolar epithelial cell line (A549) and a human monocytic cell line (THP-1). Environ Mol Mutagen 37:155–163
247. Prahalad AK, Inmon J, Dailey LA, Madden MC, Ghio AJ, Gallagher JE (2001) Air pollution particles mediated oxidative DNA base damage in a cell free system and in human airway epithelial cells in relation to particulate metal content and bioreactivity. Chem Res Toxicol 14:879–887
248. Danielsen PH, Loft S, Møller P (2008) DNA damage and cytotoxicity in type II lung epithelial (A549) cell cultures after exposure to diesel exhaust and urban street particles. Part Fibre Toxicol 5:6
249. Dellinger B, Pryor WA, Cueto R, Squadrito GL, Hegde V, Deutsch WA (2001) Role of free radicals in the toxicity of airborne fine particulate matter. Chem Res Toxicol 14:1371–1377
250. Ghio AJ, Carter JD, Dailey LA, Devlin RB, Samet JM (1999) Respiratory epithelial cells demonstrate lactoferrin receptors that increase after metal exposure. Am J Physiol 276: L933–L940
251. Albrecht C, Schins RP, Hohr D, Becker A, Shi T, Knaapen AM, Borm PJ (2004) Inflammatory time course after quartz instillation: role of tumor necrosis factor-α and particle surface. Am J Respir Cell Mol Biol 31:292–301
252. Timblin C, BeruBe K, Churg A, Driscoll K, Gordon T, Hemenway D, Walsh E, Cummins AB, Vacek P, Mossman B (1998) Ambient particulate matter causes activation of the c-jun kinase/stress-activated protein kinase cascade and DNA synthesis in lung epithelial cells. Cancer Res 58:4543–4547
253. Tamaoki J, Isono K, Takeyama K, Tagaya E, Nakata J, Nagai A (2004) Ultrafine carbon black particles stimulate proliferation of human airway epithelium via EGF receptor-mediated signaling pathway. Am J Physiol Lung Cell Mol Physiol 287:L1127–L1133
254. Sydlik U, Bierhals K, Soufi M, Abel J, Schins RP, Unfried K (2006) Ultrafine carbon particles induce apoptosis and proliferation in rat lung epithelial cells via specific signaling pathways both using EGF-R. Am J Physiol Lung Cell Mol Physiol 291:L725–L733
255. Rumelhard M, Ramgolam K, Hamel R, Marano F, Baeza-Squiban A (2007) Expression and role of EGFR ligands induced in airway cells by PM2.5 and its components. Eur Respir J 30:1064–1073
256. Baulig A, Blanchet S, Rumelhard M, Lacroix G, Marano F, Baeza-Squiban A (2007) Fine urban atmospheric particulate matter modulates inflammatory gene and protein expression in human bronchial epithelial cells. Front Biosci 12:771–782
257. Blanchet S, Ramgolam K, Baulig A, Marano F, Baeza-Squiban A (2004) Fine particulate matter induces amphiregulin secretion by bronchial epithelial cells. Am J Respir Cell Mol Biol 30:421–427
258. Camatini M, Corvaja V, Pezzolato E, Mantecca P, Gualtieri M (2012) PM10-biogenic fraction drives the seasonal variation of proinflammatory response in A549 cells. Environ Toxicol 27(2):63–73
259. Baulig A, Singh S, Marchand A, Schins R, Barouki R, Garlatti M, Marano F, Baeza-Squiban A (2009) Role of Paris PM(2.5) components in the pro-inflammatory response induced in airway epithelial cells. Toxicology 261:126–135
260. Jimenez LA, Thompson J, Brown DA, Rahman I, Antonicelli F, Duffin R, Drost EM, Hay RT, Donaldson K, MacNee W (2000) Activation of NF-κB by PM(10) occurs via an iron-mediated mechanism in the absence of IκB degradation. Toxicol Appl Pharmacol 166: 101–110
261. Fujii T, Hogg JC, Keicho N, Vincent R, Van Eeden SF, Hayashi S (2003) Adenoviral E1A modulates inflammatory mediator expression by lung epithelial cells exposed to PM10. Am J Physiol Lung Cell Mol Physiol 284:L290–L297

262. Gilmour PS, Rahman I, Hayashi S, Hogg JC, Donaldson K, MacNee W (2001) Adenoviral E1A primes alveolar epithelial cells to PM(10)-induced transcription of interleukin-8. Am J Physiol Lung Cell Mol Physiol 281:L598–L606
263. Devalia JL, Bayram H, Abdelaziz MM, Sapsford RJ, Davies RJ (1999) Differences between cytokine release from bronchial epithelial cells of asthmatic patients and non-asthmatic subjects: effect of exposure to diesel exhaust particles. Int Arch Allergy Immunol 118: 437–439
264. Gursinsky T, Ruhs S, Friess U, Diabate S, Krug HF, Silber RE, Simm A (2006) Air pollution-associated fly ash particles induce fibrotic mechanisms in primary fibroblasts. Biol Chem 387:1411–1420
265. Deng F, Guo X, Liu H, Fang X, Yang M, Chen W (2007) Effects of dust storm PM2.5 on cell proliferation and cell cycle in human lung fibroblasts. Toxicol *In Vitro* 21:632–638
266. Lindroos PM, Coin PG, Badgett A, Morgan DL, Bonner JC (1997) Alveolar macrophages stimulated with titanium dioxide, chrysotile asbestos, and residual oil fly ash upregulate the PDGF receptor-α on lung fibroblasts through an IL-1β-dependent mechanism. Am J Respir Cell Mol Biol 16:283–292
267. Bonner JC, Rice AB, Lindroos PM, O'Brien PO, Dreher KL, Rosas I, Alfaro-Moreno E, Osornio-Vargas AR (1998) Induction of the lung myofibroblast PDGF receptor system by urban ambient particles from Mexico City. Am J Respir Cell Mol Biol 19:672–680
268. Kim H, Liu X, Kobayashi T, Kohyama T, Wen FQ, Romberger DJ, Conner H, Gilmour PS, Donaldson K, MacNee W, Rennard SI (2003) Ultrafine carbon black particles inhibit human lung fibroblast-mediated collagen gel contraction. Am J Respir Cell Mol Biol 28:111–121
269. Kiama SG, Cochand L, Karlsson L, Nicod LP, Gehr P (2001) Evaluation of phagocytic activity in human monocyte-derived dendritic cells. J Aerosol Med 14:289–299
270. Porter M, Karp M, Killedar S, Bauer SM, Guo J, Williams D, Breysse P, Georas SN, Williams MA (2007) Diesel-enriched particulate matter functionally activates human dendritic cells. Am J Respir Cell Mol Biol 37:706–719
271. Williams MA, Porter M, Horton M, Guo J, Roman J, Williams D, Breysse P, Georas SN (2007) Ambient particulate matter directs nonclassic dendritic cell activation and a mixed TH1/TH2-like cytokine response by naive CD4+ T cells. J Allergy Clin Immunol 119: 488–497
272. Braun A, Bewersdorff M, Lintelmann J, Matuschek G, Jakob T, Göttlicher M, Schober W, Buters JT, Behrendt H, Mempel M (2010) Differential impact of diesel particle composition on pro-allergic dendritic cell function. Toxicol Sci 113:85–94
273. Chan RC, Wang M, Li N, Yanagawa Y, Onoe K, Lee JJ, Nel AE (2006) Pro-oxidative diesel exhaust particle chemicals inhibit LPS-induced dendritic cell responses involved in T-helper differentiation. J Allergy Clin Immunol 118:455–465
274. Viera L, Chen K, Nel A, Lloret MG (2009) The impact of air pollutants as an adjuvant for allergic sensitization and asthma. Curr Allergy Asthma Rep 9:327–333
275. Williams MA, Rangasamy T, Bauer SM, Killedar S, Karp M, Kensler TW, Yamamoto M, Breysse P, Biswal S, Georas SN (2008) Disruption of the transcription factor Nrf2 promotes pro-oxidative dendritic cells that stimulate Th2-like immunoresponsiveness upon activation by ambient particulate matter. J Immunol 181:4545–4559
276. Koike E, Takano H, Inoue K, Yanagisawa R, Kobayashi T (2008) Carbon black nanoparticles promote the maturation and function of mouse bone marrow-derived dendritic cells. Chemosphere 73:371–376
277. Bleck B, Tse DB, Jaspers I, Curotto de Lafaille MA, Reibman J (2006) Diesel exhaust particle-exposed human bronchial epithelial cells induce dendritic cell maturation. J Immunol 176:7431–7437
278. Ying S, O'Connor B, Ratoff J, Meng Q, Mallett K, Cousins D, Robinson D, Zhang G, Zhao J, Lee TH, Corrigan C (2005) Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity. J Immunol 174:8183–8190

279. Bleck B, Tse DB, Curotto de Lafaille MA, Zhang F, Reibman J (2008) Diesel exhaust particle-exposed human bronchial epithelial cells induce dendritic cell maturation and polarization via thymic stromal lymphopoietin. J Clin Immunol 28:147–156
280. Li R, Ning Z, Cui J, Khalsa B, Ai L, Takabe W, Beebe T, Majumdar R, Sioutas C, Hsiai T (2009) Ultrafine particles from diesel engines induce vascular oxidative stress via JNK activation. Free Radic Biol Med 46:775–782
281. Mo Y, Wan R, Chien S, Tollerud DJ, Zhang Q (2009) Activation of endothelial cells after exposure to ambient ultrafine particles: the role of NADPH oxidase. Toxicol Appl Pharmacol 236:183–193
282. Wei H, Wei D, Yi S, Zhang F, Ding W (2011) Oxidative stress induced by urban fine particles in cultured EA.hy926 cells. Hum Exp Toxicol 30:579–590
283. Totlandsdal AI, Refsnes M, Skomedal T, Osnes JB, Schwarze PE, Lag M (2008) Particle-induced cytokine responses in cardiac cell cultures—the effect of particles *versus* soluble mediators released by particle-exposed lung cells. Toxicol Sci 106:233–241
284. Qu S, Liberda EN, Qu Q, Chen LC (2010) *In vitro* assessment of the inflammatory response of respiratory endothelial cells exposed to particulate matter. J Toxicol Environ Health A 73:1113–1121
285. Yamawaki H, Iwai N (2006) Mechanisms underlying nano-sized air-pollution-mediated progression of atherosclerosis: carbon black causes cytotoxic injury/inflammation and inhibits cell growth in vascular endothelial cells. Circ J 70:129–140
286. Alfaro-Moreno E, Lopez-Marure R, Montiel-Davalos A, Symonds P, Osornio-Vargas AR, Rosas I, Clifford Murray J (2007) E-Selectin expression in human endothelial cells exposed to PM10: the role of endotoxin and insoluble fraction. Environ Res 103:221–228
287. Kristovich R, Knight DA, Long JF, Williams MV, Dutta PK, Waldman WJ (2004) Macrophage-mediated endothelial inflammatory responses to airborne particulates: impact of particulate physicochemical properties. Chem Res Toxicol 17:1303–1312
288. Montiel-Davalos A, Alfaro-Moreno E, Lopez-Marure R (2007) PM2.5 and PM10 induce the expression of adhesion molecules and the adhesion of monocytic cells to human umbilical vein endothelial cells. Inhal Toxicol 19(Suppl 1):91–98
289. Li R, Ning Z, Cui J, Yu F, Sioutas C, Hsiai T (2010) Diesel exhaust particles modulate vascular endothelial cell permeability: implication of ZO-1 expression. Toxicol Lett 197: 163–168
290. Wang T, Chiang ET, Moreno-Vinasco L, Lang GD, Pendyala S, Samet JM, Geyh AS, Breysse PN, Chillrud SN, Natarajan V, Garcia JG (2010) Particulate matter disrupts human lung endothelial barrier integrity via ROS- and p38 MAPK-dependent pathways. Am J Respir Cell Mol Biol 42:442–449
291. Knaapen AM, den Hartog GJ, Bast A, Borm PJ (2001) Ambient particulate matter induces relaxation of rat aortic rings *in vitro*. Hum Exp Toxicol 20:259–265
292. Miller MR, Borthwick SJ, Shaw CA, McLean SG, McClure D, Mills NL, Duffin R, Donaldson K, Megson IL, Hadoke PW, Newby DE (2009) Direct impairment of vascular function by diesel exhaust particulate through reduced bioavailability of endothelium-derived nitric oxide induced by superoxide free radicals. Environ Health Perspect 117: 611–616
293. Block ML, Wu X, Pei Z, Li G, Wang T, Qin L, Wilson B, Yang J, Hong JS, Veronesi B (2004) Nanometer size diesel exhaust particles are selectively toxic to dopaminergic neurons: the role of microglia, phagocytosis, and NADPH oxidase. FASEB J 18:1618–1620
294. Hartz AM, Bauer B, Block ML, Hong JS, Miller DS (2008) Diesel exhaust particles induce oxidative stress, proinflammatory signaling, and P-glycoprotein up-regulation at the blood-brain barrier. FASEB J 22:2723–2733
295. Sama P, Long TC, Hester S, Tajuba J, Parker J, Chen LC, Veronesi B (2007) The cellular and genomic response of an immortalized microglia cell line (BV2) to concentrated ambient particulate matter. Inhal Toxicol 19:1079–1087

296. Shin JA, Lee EJ, Seo SM, Kim HS, Kang JL, Park EM (2010) Nanosized titanium dioxide enhanced inflammatory responses in the septic brain of mouse. Neuroscience 165:445–454
297. Campbell A, Araujo JA, Li H, Sioutas C, Kleinman M (2009) Particulate matter induced enhancement of inflammatory markers in the brains of apolipoprotein E knockout mice. J Nanosci Nanotechnol 9:5099–5104
298. Harkema JR, Wagner JG, Kaminski NE, Morishita M, Keeler GJ, McDonald JD, Barrett EG (2009) Effects of concentrated ambient particles and diesel engine exhaust on allergic airway disease in Brown Norway rats. Res Rep Health Eff Inst 145:5–55
299. Mills NL, Robinson SD, Fokkens PH, Leseman DL, Miller MR, Anderson D, Freney EJ, Heal MR, Donovan RJ, Blomberg A, Sandström T, MacNee W, Boon NA, Donaldson K, Newby DE, Cassee FR (2008) Exposure to concentrated ambient particles does not affect vascular function in patients with coronary heart disease. Environ Health Perspect 116: 709–715
300. Stringer B, Imrich A, Kobzik L (1996) Lung epithelial cell (A549) interaction with unopsonized environmental particulates: quantitation of particle-specific binding and IL-8 production. Exp Lung Res 22:495–508
301. Barlow PG, Brown DM, Donaldson K, MacCallum J, Stone V (2008) Reduced alveolar macrophage migration induced by acute ambient particle (PM10) exposure. Cell Biol Toxicol 24:243–252
302. Gilmour MI, McGee J, Duvall RM, Dailey L, Daniels M, Boykin E, Cho SH, Doerfler D, Gordon T, Devlin RB (2007) Comparative toxicity of size-fractionated airborne particulate matter obtained from different cities in the United States. Inhal Toxicol 19(Suppl 1):7–16
303. Rao KM, Ma JY, Meighan T, Barger MW, Pack D, Vallyathan V (2005) Time course of gene expression of inflammatory mediators in rat lung after diesel exhaust particle exposure. Environ Health Perspect 113:612–617
304. Marano F, Boland S, Bonvallot V, Baulig A, Baeza-Squiban A (2002) Human airway epithelial cells in culture for studying the molecular mechanisms of the inflammatory response triggered by diesel exhaust particles. Cell Biol Toxicol 18:315–320
305. Salnikow K, Li X, Lippmann M (2004) Effect of nickel and iron co-exposure on human lung cells. Toxicol Appl Pharmacol 196:258–265
306. Zanchi AC, Saiki M, Saldiva PH, Barros HM, Rhoden CR (2010) Hippocampus lipid peroxidation induced by residual oil fly ash intranasal instillation *versus* habituation to the open field. Inhal Toxicol 22:84–88
307. Steerenberg PA, Withagen CE, Dormans JA, van Dalen WJ, van Loveren H, Casee FR (2003) Adjuvant activity of various diesel exhaust and ambient particles in two allergic models. J Toxicol Environ Health A 66:1421–1439
308. Smith KR, Veranth JM, Kodavanti UP, Aust AE, Pinkerton KE (2006) Acute pulmonary and systemic effects of inhaled coal fly ash in rats: comparison to ambient environmental particles. Toxicol Sci 93:390–399
309. Donaldson K, Mills N, MacNee W, Robinson S, Newby D (2005) Role of inflammation in cardiopulmonary health effects of PM. Toxicol Appl Pharmacol 207:483–488
310. Stone V, Tuinman M, Vamvakopoulos JE, Shaw J, Brown D, Petterson S, Faux SP, Borm P, MacNee W, Michaelangeli F, Donaldson K (2000) Increased calcium influx in a monocytic cell line on exposure to ultrafine carbon black. Eur Respir J 15:297–303
311. Tong H, McGee JK, Saxena RK, Kodavanti UP, Devlin RB, Gilmour MI (2009) Influence of acid functionalization on the cardiopulmonary toxicity of carbon nanotubes and carbon black particles in mice. Toxicol Appl Pharmacol 239:224–232
312. Wilson MR, Lightbody JH, Donaldson K, Sales J, Stone V (2002) Interactions between ultrafine particles and transition metals *in vivo* and *in vitro*. Toxicol Appl Pharmacol 184:172–179
313. Hussain S, Vanoirbeek JA, Luyts K, De Vooght V, Verbeken E, Thomassen LC, Martens JA, Dinsdale D, Boland S, Marano F, Nemery B, Hoet PH (2011) Lung exposure to nanoparticles modulates an asthmatic response in a mouse model of asthma. Eur Respir J 37:299–309
314. Oberdörster G, Finkelstein JN, Johnston C, Gelein R, Cox C, Baggs R, Elder AC (2000) Acute pulmonary effects of ultrafine particles in rats and mice. Res Rep Health Eff Inst 96:5–74 (discussion 75–86)

315. Singh S, Shi T, Duffin R, Albrecht C, van Berlo D, Hohr D, Fubini B, Martra G, Fenoglio I, Borm PJ, Schins RP (2007) Endocytosis, oxidative stress and IL-8 expression in human lung epithelial cells upon treatment with fine and ultrafine TiO_2: role of the specific surface area and of surface methylation of the particles. Toxicol Appl Pharmacol 222:141–151
316. Curjuric I, Imboden M, Schindler C, Downs SH, Hersberger M, Liu SL, Matyas G, Russi EW, Schwartz J, Thun GA, Postma DS, Rochat T, Probst-Hensch NM (2010) HMOX1 and GST variants modify attenuation of FEF25-75% decline due to PM10 reduction. Eur Respir J 35:505–514
317. Ren C, Baccarelli A, Wilker E, Suh H, Sparrow D, Vokonas P, Wright R, Schwartz J (2010) Lipid and endothelium-related genes, ambient particulate matter, and heart rate variability—the VA normative aging study. J Epidemiol Community Health 64:49–56
318. Wilker E, Mittleman MA, Litonjua AA, Poon A, Baccarelli A, Suh H, Wright RO, Sparrow D, Vokonas P, Schwartz J (2009) Postural changes in blood pressure associated with interactions between candidate genes for chronic respiratory diseases and exposure to particulate matter. Environ Health Perspect 117:935–940
319. Imboden M, Schwartz J, Schindler C, Curjuric I, Berger W, Liu SL, Russi EW, Ackermann-Liebrich U, Rochat T, Probst-Hensch NM (2009) Decreased PM10 exposure attenuates age-related lung function decline: genetic variants in p53, p21, and CCND1 modify this effect. Environ Health Perspect 117:1420–1427
320. Wenten M, Gauderman WJ, Berhane K, Lin PC, Peters J, Gilliland FD (2009) Functional variants in the catalase and myeloperoxidase genes, ambient air pollution, and respiratory-related school absences: an example of epistasis in gene-environment interactions. Am J Epidemiol 170:1494–1501
321. Peters A, Greven S, Heid IM, Baldari F, Breitner S, Bellander T, Chrysohoou C, Illig T, Jacquemin B, Koenig W, Lanki T, Nyberg F, Pekkanen J, Pistelli R, Rückerl R, Stefanadis C, Schneider A, Sunyer J, Wichmann HE (2009) Fibrinogen genes modify the fibrinogen response to ambient particulate matter. Am J Respir Crit Care Med 179:484–491
322. Baccarelli A, Cassano PA, Litonjua A, Park SK, Suh H, Sparrow D, Vokonas P, Schwartz J (2008) Cardiac autonomic dysfunction: effects from particulate air pollution and protection by dietary methyl nutrients and metabolic polymorphisms. Circulation 117:1802–1809
323. Park GY, Christman JW (2006) Involvement of cyclooxygenase-2 and prostaglandins in the molecular pathogenesis of inflammatory lung diseases. Am J Physiol Lung Cell Mol Physiol 290:L797–L805
324. Montiel-Davalos A, Ibarra-Sanchez Mde J, Ventura-Gallegos JL, Alfaro-Moreno E, Lopez-Marure R (2010) Oxidative stress and apoptosis are induced in human endothelial cells exposed to urban particulate matter. Toxicol In Vitro 24:135–141
325. Zhao Y, Usatyuk PV, Gorshkova IA, He D, Wang T, Moreno-Vinasco L, Geyh AS, Breysse PN, Samet JM, Spannhake EW, Garcia JG, Natarajan V (2009) Regulation of COX-2 expression and IL-6 release by particulate matter in airway epithelial cells. Am J Respir Cell Mol Biol 40:19–30
326. Dagher Z, Garcon G, Billet S, Verdin A, Ledoux F, Courcot D, Aboukais A, Shirali P (2007) Role of nuclear factor-κB activation in the adverse effects induced by air pollution particulate matter (PM2.5) in human epithelial lung cells (L132) in culture. J Appl Toxicol 27: 284–290
327. Churg A, Xie C, Wang X, Vincent R, Wang RD (2005) Air pollution particles activate NF-κ B on contact with airway epithelial cell surfaces. Toxicol Appl Pharmacol 208:37–45
328. Maciejczyk P, Chen LC (2005) Effects of subchronic exposures to concentrated ambient particles (CAPs) in mice VIII. Source-related daily variations in *in vitro* responses to CAPs. Inhal Toxicol 17:243–253
329. Nam HY, Choi BH, Lee JY, Lee SG, Kim YH, Lee KH, Yoon HK, Song JS, Kim HJ, Lim Y (2004) The role of nitric oxide in the particulate matter (PM2.5)-induced NFκB activation in lung epithelial cells. Toxicol Lett 148:95–102
330. Shukla A, Timblin C, BeruBe K, Gordon T, McKinney W, Driscoll K, Vacek P, Mossman BT (2000) Inhaled particulate matter causes expression of nuclear factor (NF)-κB-related

genes and oxidant-dependent NF-κB activation *in vitro*. Am J Respir Cell Mol Biol 23: 182–187
331. Li R, Ning Z, Majumdar R, Cui J, Takabe W, Jen N, Sioutas C, Hsiai T (2010) Ultrafine particles from diesel vehicle emissions at different driving cycles induce differential vascular pro-inflammatory responses: implication of chemical components and NF-κB signaling. Part Fibre Toxicol 7:6
332. Tal TL, Simmons SO, Silbajoris R, Dailey L, Cho SH, Ramabhadran R, Linak W, Reed W, Bromberg PA, Samet JM (2010) Differential transcriptional regulation of IL-8 expression by human airway epithelial cells exposed to diesel exhaust particles. Toxicol Appl Pharmacol 243:46–54
333. Totlandsdal AI, Refsnes M, Lag M (2010) Mechanisms involved in ultrafine carbon black-induced release of IL-6 from primary rat epithelial lung cells. Toxicol In Vitro 24:10–20
334. Alessandrini F, Beck-Speier I, Krappmann D, Weichenmeier I, Takenaka S, Karg E, Kloo B, Schulz H, Jakob T, Mempel M, Behrendt H (2009) Role of oxidative stress in ultrafine particle-induced exacerbation of allergic lung inflammation. Am J Respir Crit Care Med 179:984–991
335. Hirota R, Akimaru K, Nakamura H (2008) *In vitro* toxicity evaluation of diesel exhaust particles on human eosinophilic cell. Toxicol In Vitro 22:988–994
336. Mroz RM, Schins RP, Li H, Drost EM, Macnee W, Donaldson K (2007) Nanoparticle carbon black driven DNA damage induces growth arrest and AP-1 and NFκB DNA binding in lung epithelial A549 cell line. J Physiol Pharmacol 58(Suppl 5):461–470
337. Lee CC, Cheng YW, Kang JJ (2005) Motorcycle exhaust particles induce IL-8 production through NF-κB activation in human airway epithelial cells. J Toxicol Environ Health A 68: 1537–1555
338. Yun YP, Joo JD, Lee JY, Nam HY, Kim YH, Lee KH, Lim CS, Kim HJ, Lim YG, Lim Y (2005) Induction of nuclear factor-κB activation through TAK1 and NIK by diesel exhaust particles in L2 cell lines. Toxicol Lett 155:337–342
339. Ma C, Wang J, Luo J (2004) Activation of nuclear factor κB by diesel exhaust particles in mouse epidermal cells through phosphatidylinositol 3-kinase/Akt signaling pathway. Biochem Pharmacol 67:1975–1983
340. Roberts E, Charboneau L, Espina V, Liotta L, Petricoin E, Dreher K (2004) Application of laser capture microdissection and protein microarray technologies in the molecular analysis of airway injury following pollution particle exposure. J Toxicol Environ Health A 67: 851–861
341. Takizawa H, Abe S, Okazaki H, Kohyama T, Sugawara I, Saito Y, Ohtoshi T, Kawasaki S, Desaki M, Nakahara K, Zamamoto K, Matsushima K, Tanaka M, Sagai M, Kudoh S (2003) Diesel exhaust particles upregulate eotaxin gene expression in human bronchial epithelial cells via nuclear factor-κB-dependent pathway. Am J Physiol Lung Cell Mol Physiol 284: L1055–L1062
342. Zhou YM, Zhong CY, Kennedy IM, Leppert VJ, Pinkerton KE (2003) Oxidative stress and NFκB activation in the lungs of rats: a synergistic interaction between soot and iron particles. Toxicol Appl Pharmacol 190:157–169
343. Zhou YM, Zhong CY, Kennedy IM, Pinkerton KE (2003) Pulmonary responses of acute exposure to ultrafine iron particles in healthy adult rats. Environ Toxicol 18:227–235
344. Samet JM, Silbajoris R, Huang T, Jaspers I (2002) Transcription factor activation following exposure of an intact lung preparation to metallic particulate matter. Environ Health Perspect 110:985–990
345. Kawasaki S, Takizawa H, Takami K, Desaki M, Okazaki H, Kasama T, Kobayashi K, Yamamoto K, Nakahara K, Tanaka M, Sagai M, Ohtoshi T (2001) Benzene-extracted components are important for the major activity of diesel exhaust particles: effect on interleukin-8 gene expression in human bronchial epithelial cells. Am J Respir Cell Mol Biol 24:419–426
346. Takizawa H, Ohtoshi T, Kawasaki S, Kohyama T, Desaki M, Kasama T, Kobayashi K, Nakahara K, Yamamoto K, Matsushima K, Kudoh S (1999) Diesel exhaust particles induce

NF-κB activation in human bronchial epithelial cells *in vitro*: importance in cytokine transcription. J Immunol 162:4705–4711
347. Drumm K, Messner C, Kienast K (1999) Reactive oxygen intermediate-release of fibre-exposed monocytes increases inflammatory cytokine-mRNA level, protein tyrosine kinase and NF-κB activity in co-cultured bronchial epithelial cells (BEAS-2B). Eur J Med Res 4:257–263
348. Oettinger R, Drumm K, Knorst M, Krinyak P, Smolarski R, Kienast K (1999) Production of reactive oxygen intermediates by human macrophages exposed to soot particles and asbestos fibers and increase in NF-κB p50/p105 mRNA. Lung 177:343–354
349. Drumm K, Oettinger R, Smolarski R, Bay M, Kienast K (1998) *In vitro* study of human alveolar macrophages inflammatory mediator transcriptions and releases induced by soot FR 101, Printex 90, titandioxide and Chrysotile B. Eur J Med Res 3:432–438
350. Quay JL, Reed W, Samet J, Devlin RB (1998) Air pollution particles induce IL-6 gene expression in human airway epithelial cells via NF-κB activation. Am J Respir Cell Mol Biol 19:98–106
351. Watterson TL, Sorensen J, Martin R, Coulombe RA Jr (2007) Effects of PM2.5 collected from Cache Valley Utah on genes associated with the inflammatory response in human lung cells. J Toxicol Environ Health A 70:1731–1744
352. Ramage L, Guy K (2004) Expression of C-reactive protein and heat-shock protein-70 in the lung epithelial cell line A549, in response to PM10 exposure. Inhal Toxicol 16:447–452
353. Reibman J, Hsu Y, Chen LC, Kumar A, Su WC, Choy W, Talbot A, Gordon T (2002) Size fractions of ambient particulate matter induce granulocyte macrophage colony-stimulating factor in human bronchial epithelial cells by mitogen-activated protein kinase pathways. Am J Respir Cell Mol Biol 27:455–462

Nanomaterials: A Challenge for Toxicological Risk Assessment?

Andrea Haase, Jutta Tentschert, and Andreas Luch

Abstract Nanotechnology has emerged as one of the central technologies in the twenty-first century. This judgment becomes apparent by considering the increasing numbers of people employed in this area; the numbers of patents, of scientific publications, of products on the market; and the amounts of money invested in R&D. Prospects originating from different fields of nanoapplication seem unlimited. However, nanotechnology certainly will not be able to meet all of the ambitious expectations communicated, yet has high potential to heavily affect our daily life in the years to come. This might occur in particular in the field of consumer products, for example, by introducing nanomaterials in cosmetics, textiles, or food contact materials. Another promising area is the application of nanotechnology in medicine fueling hopes to significantly improve diagnosis and treatment of all kinds of diseases. In addition, novel technologies applying nanomaterials are expected to be instrumental in waste remediation and in the production of efficient energy storage devices and thus may help to overcome world's energy problems or to revolutionize computer and data storage technologies. In this chapter, we will focus on nanomaterials. After a brief historic and general overview, current proposals of how to define nanomaterials will be summarized. Due to general limitations, there is still no single, internationally accepted definition of the term "nanomaterial." After elaborating on the status quo and the scope of nanoanalytics and its shortcomings, the current thinking about possible hazards resulting from nanoparticulate exposures, there will be an emphasis on the requirements to be fulfilled for appropriate health risk assessment and regulation of nanomaterials. With regard to reliable risk assessments, until now there is still the remaining issue to be resolved of whether or not specific challenges and unique features exist on the nanoscale that have to be tackled and distinctively addressed, given that they

Andrea Haase, Jutta Tentschert and Andreas Luch (✉)
Department of Product Safety, German Federal Institute for Risk Assessment (BfR),
Max-Dohrn-Strasse 8-10, 10589 Berlin, Germany
e-mail:Andreas.Luch@bfr.bund.de

A. Luch (ed.), *Molecular, Clinical and Environmental Toxicology*,
Experientia Supplementum 101, DOI 10.1007/978-3-7643-8340-4_8,

substantially differ from those encountered with microsized materials or regular chemicals. Based on the current knowledge, we finally provide a proposal on how risk assessment in the nanofield could be achieved and how it might look like in the near future.

Keywords Agglomeration · Aggregation · Bioavailability · Coating · Consumer products · Inhalation exposure · *In vitro* · *In situ* analytics · *In vitro* · *In vivo* · Nanoanalytics · Nano-bio interface · Nanocomposites · Nanomedicine · Nano-objects · Nanoparticles · Nanoparticle space · Nanosensors · Nanosystems · Nanotoxicology · Particle corona · Quantum dots · Read-across · Skin penetration · Structure–(re)activity relationship · Surface plasmonics · UV filters · Waste remediation

Introduction

Rarely any technology has gained such a tremendous scientific and economic interest within such a short period of time. Although it looks that the first hype on nanotechnology is already gone, the annual investment in this technology still is increasing albeit with smaller slope [1, 2]. It is often stated that nanotechnology will be the "Technology of the 21st century," which is supposed to influence our daily life and lead to an industrial revolution within a short time frame. In the workshop report "Nanotechnology Research Directions" from the Interagency Working Group on Nanoscience, Engineering and Technology (IWGN) of the National Science and Technology Council (NSTC), drafted in 1999, it is stated that "nanotechnology will be a strategic branch of science and engineering for the next century, one that will fundamentally restructure the technologies currently used for manufacturing, medicine, defense, energy production, environmental management, transportation, communication, computation, and education" [3, 4].

So, nanotechnology looks like a very young technology. Nevertheless, nanomaterials actually existed, were synthesized, and used long before the term nanotechnology was coined [5, 6]. The size of the particles usually serves as main criterion to define what a nanoparticle is supposed to be. The properties of nanomaterials are very different compared to their bulky counterparts. This may refer to physical, chemical, or electrical properties such as extraordinary strength or highly advanced optical or catalytical properties. This is what makes nanomaterials so interesting for a wide range of application fields. As the use of nanomaterials is steadily increasing and many products furnished with nanotechnology are released to the market, the concerns about the safety of this technology and about possible risks for humans and the environment gain heavy weight as well. Certainly, while the majority of the investment is still spent on basic science such as for the development of new materials, the awareness of a proper safety assessment has led to increased efforts

in the development of toxicological assays, in the advancement of exposure monitoring measures, as well as in the development of risk assessment strategies. Partially this is also due to undesirable developments in other fields, such as genetically modified organisms, where fearful public perception and unsubstantiated concerns strongly opposed the industrial use and further development of this technology. From the very beginning, in the nanotechnology field, a substantial amount of money was invested in safety aspects and in the understanding of the public perception and awareness about this technology, as well as in communication strategies [7].

Much more than in any other scientific field, the development and advancement of nanotechnology strongly depend on interdisciplinary cooperation. Expert knowledge is needed from material sciences, physics, analytics, chemistry, pharmacy, biology, medicine, toxicology, and many more highly specialized subdisciplines. Here, we want to provide a glimpse on the history of nanotechnology, to explain the issues related to definitions, and to introduce the extraordinary properties of nanomaterials and their current and possible future application fields. We will focus on "nanoanalytics" and "nanotoxicology" and how these areas could be reasonably combined. This chapter is not intended to comprehensively cover all topics related to nanotechnology. Rather, we will provide an overview on issues related to risk assessment and otherwise refer the interested reader to the excellent literature in this field (e.g., [8–12]). It should be already emphasized at this point that future efforts should be focused on *in situ* analytics and toxicological characterization. These fields are just about to emerge and might be the key for the development of structure–activity relationships applicable in health risk assessment.

History of Nanotechnology

Nanotechnology existed long before people knew about nanoparticles. Already in the fifth century B.C., colloidal gold was known in Egypt or China and, for instance, applied in medicine [13]. In roman times, nanosized silver and gold particles were used to paint glass; a well-known example is "The Lycurgus Cup." This application was also very popular in medieval times as the wonderful colored church windows at many places still allow us to recognize. For a long time the chemical nature of the applied gold and silver preparations was unknown. It was only speculation that these preparations contained gold "in such a degree of communition that it is not visible to the human eye" as Johann Kunckel wrote in 1679 [14]. In 1857, Michael Faraday was the first to synthesize tiny, nanosized gold particles intentionally by reduction of gold chloride ($AuCl_4^-$) [15]. This was the beginning of colloidal sciences. Although both terms are not synonymous and not all nanomaterials are colloids and *vice versa*, still what we call nanoscience today has its origins in the science of colloids [16]. The concept about what is possible at the nanoscale and thus the first concept of nanotechnology was introduced in 1959 by the Nobel laureate Richard Feynman in his famous lecture "There is plenty of room at the

bottom" [17]. He explained: "The principles of physics as far as I can see, do not speak against the possibility of maneuvering things atom by atom." He was also the first to use the word "nanostructures." At this time, it was still a more philosophical or theoretical problem. The technologies to manipulate and analyze materials at the nanoscale still had first to be developed. Feynman speculated about exciting new discoveries which would be possible at the nanoscale. But only with the invention of the scanning tunneling microscope (STM) in 1981 or the atomic force microscopy (AFM) in 1986, there was a possibility to understand materials far down to the nanoscale. So, truly nanotechnology in a sense of understanding that we share today was actually emerging in the second half of the twentieth century [18, 19]. The term "nanotechnology" was first used in 1974 by the Japanese university professor Norio Taniguchi [20]. In the 1970s, the idea of using nanoparticles as drug delivery systems became very popular, and substantial work in this field was done by Kreuter and coworkers [21]. Up to now, nanomedicine is still one of the main and most fascinating fields of nanoscience.

As a general consensus, the term nanoparticle or nanomaterial should only be used for intentionally produced materials. Nanoparticles can also be formed unintentionally or incidentally either by human activities (e.g., during combustion processes) or naturally. Such particles can be found in aquatic and terrestrial environments as well as in the atmosphere. At several ocean sides (e.g., around Cape Horn or at the West Coast of Ireland), significant numbers of particles in the size range of 10–100 nm were detectable, but also in other natural environments, such as the boreal forest Hyytiälä (Southern Finland), high numbers of nanoparticles of natural origin have been measured [22]. So, a significant proportion of the naturally occurring colloids are nanosized. They are heterogenous in size, shape, chemical composition, and property. They may be inorganic (e.g., mostly based on aluminum phyllosilicates or iron oxides/hydrous ferric oxides) or organic (e.g., so-called naturally occurring matter) [23, 24]. Furthermore, several biostructures belong to this size range. A typical protein is around 4–6 nm in size, and protein assemblies such as ribosomes are nanoscaled. A typical virus which is about 100 nm in size clearly belongs to the nanostructured world, while bacteria ($\geq$1 μm) usually do not.

Definitions

Nanoscience is the science that deals with materials at the nanoscale, meaning to synthesize, manipulate, or study nanoscaled materials. According to most definitions, the nanoscaled world covers the size range between approximately 1 and 100 nm [25]. Nanotechnology is defined as "the research and technology development at the atomic, molecular or macromolecular levels, in the length scale of approximately 1–100 nm; the creation, and use of structures, devices and systems that have novel properties and functions because of their small size; and ability to be controlled or manipulated on the atomic scale" [26]. Similar definitions exist

from the International Organization for Standardization (ISO; http://www.iso.org/iso/home.htm) and other international or national bodies. Nanomaterials, on the other hand, are defined as materials that are—at least in one dimension (external or internal)—in the size range between 1 and 100 nm. However, there are also few exceptions from this size range known, such as, for instance, graphene, which is made of carbon sheets thinner than 1 nm [27]. Due to their tiny size, nanomaterials have novel, so-called nanospecific, properties that differ from those of the same material at larger sizes. Indeed, most of the definitions for nanomaterials are based solely or mainly on its size. According to this, the "nanoworld" covers the range between the world of atoms and molecules on one side and the world of bulk material at the other. However, it should be kept in mind that there is still no comprehensive definition that would be generally and internationally accepted and legally binding; current efforts are great to reach an internationally harmonized version [28].

According to the European Committee for Standardization (CEN) Technical Specification 27687 [29], nanomaterials encompass nanoobjects and nanostructured materials (Fig. 1). Nanoobjects are nanoscaled at least in one dimension; this might be the case just for one external dimension (i.e., nanoplates), for two (i.e., nanorods), or for all three dimensions (i.e., nanoparticles). Nanorods can be further separated into nanofibers (flexible nanorods), nanotubes (hollow nanofibers), or nanowires (electrically conducting or semiconducting materials). On the other hand, nanostructured materials may contain either nanocomposites or nanoparticles, or they display nanostructured surfaces (Fig. 1).

Recent discussions in the ISO Technical Committee (TC) working group TC 229/JWG 1 focusing on "Terminology and nomenclature" (http://www.iso.org/iso/iso_technical_committee?commid=381983) even suggest that the term "nanomaterial" should be replaced by "nanoparticulate material" to avoid misunderstandings. Furthermore, TC 229/JWG 1 proposed to include an additional subgroup

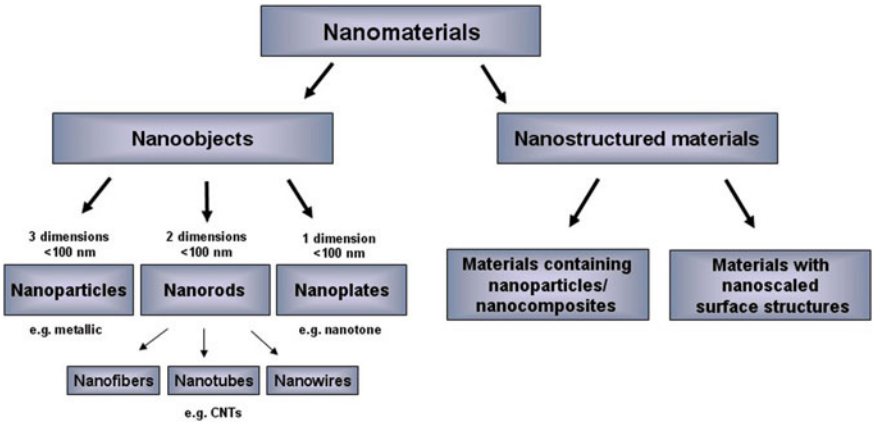


Fig. 1 Different nanomaterials according to CEN/ISO TS 27687 [29]

among nanoobjects, which could represent the novel, more complex forms of nanomaterials like core–shell structures that recently gained interest due to their multifunctionality.

Importantly, the size limits given are just approximative rather than absolute. Since nanospecific properties of the material do not necessarily follow exactly the size limits used for defining what "nano" is, the size-based definition alone may entail several drawbacks. In addition, the size of nanoparticles always spans a whole range and follows individual distribution curves. So, the so-called primary particle size always represents a mean value, and each type of nanoparticle displays its own polydispersity. As a consequence, even batches of nanoparticles with a mean size of $>$100 nm contain a certain fraction of particles below 100 nm.

Another issue is the likely formation of agglomerates and aggregates. Nanoparticles intrinsically tend to form agglomerates (due to rather loose interactions between individual particles; e.g., based on van der Waals forces) and aggregates (due to strong interactions between individual particles, resulting in fused or sintered particles) [29]. Some nanospecific properties such as a large surface-to-volume ratio might also retain in agglomerates and aggregates, and over time single primary particles might be released from it. Occasionally, it has been proposed to include this fact and to specify a particular nanomaterial based on the fraction (in percent) of free and individual nanoparticles it contains [30]. According to this, a nanomaterial contains particles in the size range of approximately 1–100 nm at more than x% of the total number size distribution; this means that the remaining part might be larger or aggregated. Currently, there is an ongoing and open discussion about the exact number of x [30].

While some definitions mainly focus on the size of the particles regardless of whether or not these particles exhibit so-called nanospecific properties, others concentrate on novel characteristics of the material which should be clearly different from the properties of the corresponding bulk material [31]. For instance, the latter definition has been proposed and disseminated by the British Standards Institution (BSI) in its Publicly Available Specifications (PAS) 71:2005 and 136:2007, stating that—by definition—a nanomaterial should "exhibit novel characteristics compared to the same material without nanoscale features" [32, 33]. However, neither these novel properties can be predicted or deduced from the corresponding bulk material, nor are they comparable to the physicochemical behavior of the corresponding (underlying) atoms or molecules. These nanospecific properties can pertain, but are not restricted to chemical reactivities, catalytic properties, electrical conductivity, optical or spectrophotometric characteristics, and so forth [34–37]. Some of these alterations are caused by the exponential increase of the specific surface area following decreasing sizes, the higher probability and impact of surface crystal defects, and the increasing likelihood of an incomplete coordination (bonding) of atoms at the particle surface [37]. All of these factors might contribute to the higher reactivity or catalytic activity of the respective material. In addition, due to an extremely small size, completely new features may appear. This refers to the occurrence of quantum effects such as, for instance, quantum confinement, which accounts for the special feature of so-called quantum

dots [38]. Such features do not emerge gradually with decreasing sizes but rather occur suddenly below a certain threshold. Other examples of nanospecific phenomena are wavelike transports, the predominance of interfacial phenomena, and the occurrence of surface plasmon resonance (SPR) in the case of, e.g., nanosilver or nanogold [39].

The term "nanospecific" property might be misleading as it implicates that nanomaterials as such display common properties that arise just because of their "nano" size. Rather, these properties strongly depend on the kind of material, on its actual size, but also on possible coatings and/or stabilizers applied. For instance, it has been demonstrated that the photocatalytic activity of molybdenum disulfide (MoS_2) depends on the particle size [40], and the properties of CdSe quantum dots are influenced by the coating [41]. It should be further noticed that the kind of coating also strongly affects the dissolution of soluble nanoparticles, as has been shown in the case of nanosilver [42]. Thus, nanospecific properties usually change as the size of the material changes. A given material of 80-nm size might have completely different features compared to exactly the same type of material at 10 nm or 2 nm. Currently, it is impossible to predict/deduce such properties or to describe them via math modeling. Approaches such as grouping of nanoparticles based upon similar composition and read-across, that is, the usage of toxicological data from one type of nanomaterial to predict the effects of another similar type, seem unfeasible at the moment. Nevertheless, the number of different nanomaterials is steadily increasing, and different approaches exist for their classification. Table 1 gives an overview on the most common types currently available.

The summary provided in the frame of this chapter is only a small part of what might be possible in future by means of nanotechnology. Others, more sophisticated

Table 1 Overview of the most common nanoobjects/nanoparticles currently applied (modified according to [13])

Carbon-based nanomaterials	Carbon black nanoparticles	
	Carbon nanotubes (CNTs)	Single-walled (SWCNT), multiwalled (MWCNT)
	Fullerene type (C_n)	$n = 60$, 70, or higher
Metal nanoparticles	Metal nanoparticles	For example, silver, gold nanoparticles
	Quantum dots	Classified according to core and shell composition [core: usually a metal and a p-block element (e.g., CdSe); shell: e.g., ZnS]
Metal oxides	Many different types	For example, TiO_2, ZnO, CeO_2, SiO_2, Fe_2O_3
Polymer type	Dendrimer type	Highly branched structures, based on polyamidoamines (PAMAMs)
	PLGA or PLA–PGA type, *etc.*	poly-D,L-lactide-co-glycolide, poly-lactide acid, poly-L-glutamic acid, *etc.*
	Polysaccharide type	Nanocellulose
Other types (selection)	Core–shell structures	
	Surface functionalized structures	
	Micelles, liposomes	

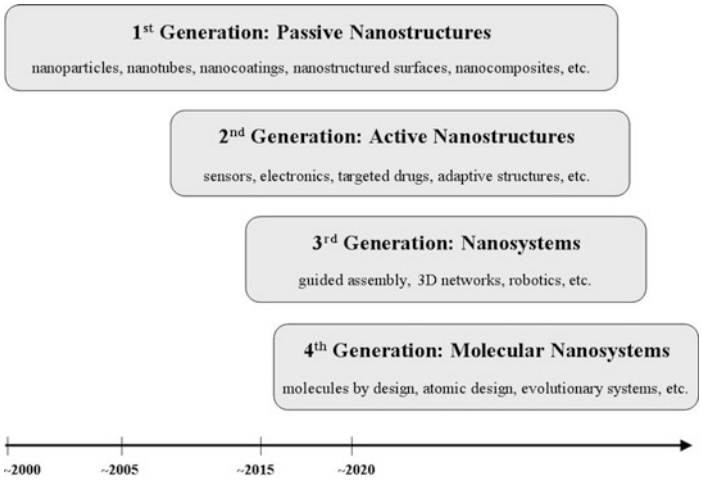


Fig. 2 Anticipated generations of nanomaterials (according to [43])

materials, are currently being the matter of R&D or are anticipated of being produced in near future. Roco predicted four overlapping generations in the advancement of nanosystems (Fig. 2) [43]. The first generation consists of passive nanostructures such as nanoparticles, nanotubes, nanocomposites, nanostructured surfaces, *etc*. Thus, the most currently used nanomaterials belong to this first generation. The second generation is made of active nanomaterials, that is, materials capable of changing their properties (e.g., shape, color, mechanical, or electrical properties) while being in use. Such materials already exist and some are also sufficiently matured for commercial application in the near future. Examples for this are sensors or drug transport systems [44].

The expected third generation will be made of nanosystems capable of self-assembly and of forming networks at the nanoscale. In the nanodevelopmental scheme proposed by Roco (Fig. 2), the fourth generation will consist of nanomaterials synthesized according to individual molecular design, combining and assembling atom by atom as already speculated by Feynman (cf. above). Regardless whether this anticipated development might become true or whether the indicated time line might be correct, the further developments of nanomaterials will continue, and many more sophisticated materials will be developed and used in all kinds of products of the future market.

Properties, Fields of Application, Benefits, Concerns

One of the most obvious characteristics of a nanoparticle is its tiny size and—in direct connection to this—the increase in its specific surface (Fig. 3). As the size of the particle drops below 10 nm, there is an exponential increase in the specific

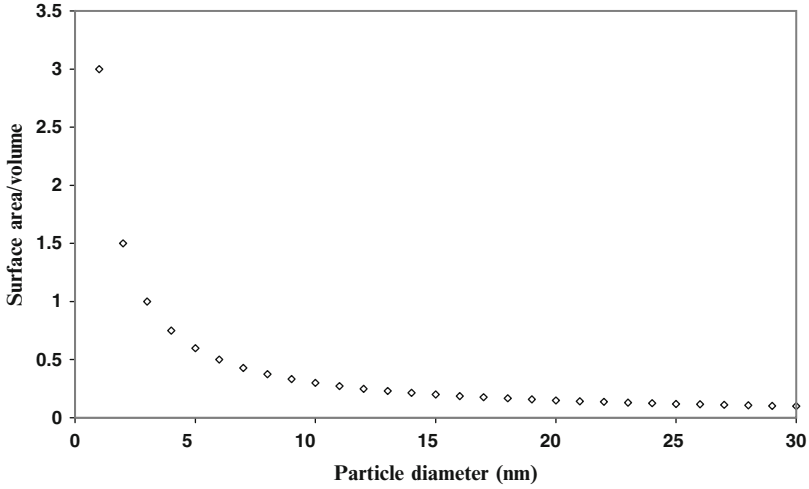


Fig. 3 Relationship between particle size (diameter) and specific surface (surface area per volume)

surface, that is, the ratio of surface to volume. As a consequence, the relative fraction of atoms located directly at the surface of the particle is significantly increasing in very small particles. While only about 0.2% of all atoms are at the surface of a 1-μm sized particle, this number increases as follows (in the case of nanogold): 3% for a 50 nm particle → 16% for a 10 nm particle → 53% for a 2.5 nm particle [37]. Therefore, surface and interfacial chemistry will be much more relevant for the understanding of nanoparticle reactivity when compared to the reactivity of the corresponding bulk material.

The chemistry of atoms at the surface clearly differs from those at the core of the particle. This is due to several reasons. In a typical crystal, there is a crystal lattice, which—in good approximation—can be assumed being infinite for bulk material. Also, each atom of a molecule has a characteristic coordination, such as, for instance, the tetrahedral coordination of saturated carbon atoms. Due to their small size and surface bending, there is no infinite lattice in nanoparticles anymore. Instead, there is a higher reactivity on the surface, a higher probability and weight of surface crystal defects, and an increased chance of undercoordination of atoms at the surface [37]. Such surface-specific chemistry is also known for bulk materials. However, due to the much higher surface/volume ratio of nanomaterials, these effects are more pronounced and decisive for particle's chemistry and physico-chemical behavior. So, all of this might contribute to the higher reactivity or to the catalytic properties of nanomaterials. A quite well-known example is the unusually high reactivity of nanogold, compared to bulk material which reveals rather inert. As a result, nanosized gold (below 10 nm in diameter) proves as excellent catalyzer, for instance, in the reduction of organic chemicals [45, 46].

Other characteristics to be taken into consideration result from the fact that nanosized particles are never "naked." Although—in theory—very small particles

might form stable suspensions without the need of further surface modification, in reality even very small particles would aggregate over time. Thus, typically, each nanoparticle requires stabilization by a certain kind of coating (either noncovalently or covalently attached to the surface) [37]. In the case of nanosilver, usually a layer of citrate or a polymer is used. Dispersions of nanoparticles principally can be stabilized via repelling surface charges (e.g., citrate coating) or via steric hindrance (e.g., polymer coating) [37]. The charging of the particle's surface, as resulting from citrate coating, induces the formation of an oppositely charged layer of ions in the dispersion medium ("Stern layer"). It seems obvious that such a charge stabilization depends on several factors, such as pH value, ionic strength of the dispersion medium, and particle concentration. The theory behind this charge stabilization has been described by Derjaguin, Landau, Verwey, and Overbeek already a while ago and became famous as "DLVO theory" (according to their first initials) [47]. Due to the wide range of influencing parameters, it usually becomes difficult to prepare highly concentrated nanoparticle stock dispersions. Also, if the stabilization of particles results from sterically demanding surface modifications, this will not lead to a universally resistant preparation either. For instance, if the particle concentration in the suspension will decrease beyond a certain threshold (below the critical micellar concentration), the polymer—if not covalently bound—might be detached and dispersed and thus all stabilizing effects will be gone [48]. To summarize, regardless by which means nanoparticles will be stabilized in suspension, an absolute stability and resistance so far remains unreachable. Instead, the fate of the suspension will depend on a range of different factors including temperature, pH, ionic strength, media composition, and others. So, over time agglomeration and aggregation of nanoparticulate suspensions is an inevitable and typical outcome.

As already mentioned above, due to their extremely small size, completely new features of nanoparticles may appear. A well-known example is the occurrence of surface plasmonics in case of nanosilver or nanogold [39]. Similar as with other physicochemical properties, the SPR depends on the surface chemistry, the shape, and the size of the nanoparticles. It also will change dramatically upon aggregation and usually disappear if all nanoparticles are engaged in aggregates. Another example is quantum confinement [38]. To briefly explain this effect at the nanoscale, we have to remember what happens when atoms form a molecule. A set of bonding and antibonding molecular orbitals (MOs) form, but all electrons are placed into the bonding MOs while the antibonding MOs remain unoccupied. In a large atomic lattice (e.g., in case of metals or semiconductors), and due to their overlap, these orbitals constitute pseudo-continua called the valence band and the conduction band. For semiconductors between both, there is a band gap. As already mentioned above, in the case of nanoparticles, there is no infinite atomic lattice and—as a result—there is a loss of orbitals that could contribute to both the valence and conduction band and thus the band gap becomes larger. If an electron, by absorption of energy, is lifted from the valence to the conduction band, it can either generate electricity or recombine by releasing (emitting) light. In the case of nanoparticles, the electrons are confined within the particle, and therefore, the

probability of light emission is much higher than the occurrence of electricity [38]. Thus, the quantum efficiency (fraction of emitted light per excitation) usually is very high. This explains the properties of so-called quantum dots, which constitute a very well-suited tool for fluorescent labeling of all kinds of materials [49].

Nanomedicine

This explanation immediately leads us to the application fields. One among these with highest expectations is nanomedicine. Here, nanomaterials are used in wound dressings, for bone cements, as coatings for prostheses or surgical instruments, in catheter tubing, in imaging contrast agents for diagnostics, but also as nanomedicine *sensu stricto*, such as, for instance, enhanced chemotherapeutics [50, 51]. Through packaging of anticancer drugs into nanoparticulate vessels, higher therapeutic doses at target organs can be reached, and unwanted toxicity and side effects may be reduced simultaneously as well. For instance, the long known and widely applied anticancer drug paclitaxel has been enclosed into aluminum nanoparticle cages and traded as Abraxane^{TM} [52]. In its conventional form, paclitaxel is difficult to formulate due to its insolubility in aqueous media; therefore, additional side effects may result from conventional formulation aids. By packaging into nanoparticles, the solubility of paclitaxel is no problem anymore. Packed into small vesicles, the drug can easily penetrate into tumor tissue which usually contains leaky, fenestrated blood vessels. By contrast, healthy tissues remain unaffected since blood is supplied through vessels with intact endothelial barriers.

Another very fascinating opportunity of nanomedicine is the use of new application forms based on novel formulations (e.g., inhalable nanoaerosols) or to better reach organs such as the brain which are shielded by specific blood–tissue barriers [53, 54]. In contrast to other application fields suitable for nanotechnology, nanomedicine relates to medicine and healthcare products and, thus, is very strictly regulated and monitored.

Consumer Products

Nanomaterials are also used in many daily-life products such as cosmetics, textiles, food contact materials, and houseware goods [55]. In the cosmetics sector, “nano” is applied in two different forms: as nanoemulsion or via incorporation of nanoobjects/nanoparticles. Nanoemulsions, meaning the encapsulation of vitamins or other sensitive compounds into liposomal cages, are prepared to facilitate the uptake of these ingredients into the body. They have reached some history yet, and this type of application is generally regarded as safe [56]. Another example is the use of nanosized titanium dioxide (TiO_2) or zinc oxide (ZnO) as UV filters in sunscreens; these metal oxides are among the most commercialized nanoparticles. Table 2

Table 2 Examples of nanoparticles used in cosmetics or textiles (some are currently in the R&D phase) (according to [57, 58])

	Particle type	Purpose
Cosmetics	TiO_2 or ZnO	UV protection
	Silver	Anti-bacterial (e.g., in deodorants)
	Fullerenes (C60)	Anti-oxidant, radical scavenging creams
	Pigments	Coloring
	Silica	Absorbance of oil, long-lasting cosmetics
	Hydroxylapatite	Tooth paste (remineralizing)
	Liposomes	Supply of, e.g., vitamins
Textiles	Silver	Anti-bacterial
	ZnO or TiO_2	UV protection
	TiO_2 or MgO	Self-sterilizing (chemical, biological protection)
	SiO_2, Al_2O_3 with special coating	Water repellent
	Ceramic	Abrasion resistance
	Nanoclay	Electrical, heat, thermal resistance
	Nanocellulose	Anti-wrinkle
	Ferrum or others	Functional textiles (e.g., conductive properties)
	Carbon nanotubes (CNTs)	Stronger fibers

summarizes the nanoparticles which are mostly used or intended to be used in different consumer products [57, 58].

For the textile sector, the best-known example is the application of antimicrobial nanosilver in or on the surface of textile fibers used to produce, for instance, sportswear, underwear, T-shirts, and socks [58]. For the same reason, nanosilver is used in coatings of household products such as washing machines or other devices. One recent and new application is the use of nanomaterials in water treatment or filtering technology, mostly carbon nanotubes (CNTs; mechanical filter) or titanium dioxide (TiO_2, photocatalytic activity) [59, 60].

Environmental Applications

Some final examples should be given for the environmental application fields. Due to their high surface binding capacity and reactivity, nanoparticles may be useful in the removal of potentially dangerous chemicals and thus applicable in waste remediation. For instance, zero-valent iron nanoparticles turned out to be useful in the removal of arsenic from groundwater [61]. Meanwhile, iron nanoparticles are also widely applied in the decontamination of soil. Different types of dendrimers can act as chelators to bind metal ions like copper, silver, or iron [62, 63]. Other applications are nanosensors, which can sense dangerous compounds in water, air, or other surroundings. Examples are tin dioxide (SnO_2)-based gas sensors or TiO_2-based electrodes to detect chemical oxygen demand [64, 65]. The use of nanotechnology often allows to produce items with less material, which in turn may be also considered environmentally beneficial. An example is the use of nanocomposites in aircrafts or cars, which have improved mechanical properties while being much

lighter than other materials applicable in this field [66]. As a consequence, fuel can be saved and less CO_2 will be emitted. Less material is also needed, for instance, with nano-based paintings or coatings; the layers applied are thinner, and thus, material can be saved.

Concerns

Concerns about the safety of nanomaterials are high [67]. One major concern arises from the high surface binding capacity of such materials. Nanoparticles can effectively bind to other potentially toxic compounds and thus may change their bioavailability as transport vehicles within living organisms [68]. Further, nanomaterials as such may be also inherently toxic. Mainly an enhanced reactivity combined with its small size, the latter allowing the particles to reach otherwise sheltered parts of the body, is the basis of such concern [69, 70]. Currently, the data on the toxicity are by far not sufficient and resilient, as we will describe in detail below. Most of the current data are from *in vitro* or acute toxicity studies and little is known about chronic toxicity endpoints [71]. At the same time, there is also concern that novel biological responses need to be considered that, by now, we even might not be able to imagine and to anticipate. This is in principle true for any novel type of chemical compound or material. The major concern in the case of nanomaterials, however, is the enormous diversity of existing materials and the endless "nanoparticle space" which is expected to be developed and produced. A conventional testing strategy that would propose each type of material to be tested individually certainly is doomed to fail. On the other hand, currently, any kind of structure–(re)activity relationship or read-across approach seems likely unreliable and dicey. To establish such tools also in the nanotoxicology field, first of all, we need to properly and fully characterize all kinds of materials under consideration. The aim will be to learn more about the material composition and its physicochemical features that are likely to contribute or to influence the biological fate and the toxicological properties of the material once it has reached and intruded living tissues and cells. For this purpose, of course, we need to establish highly advanced and reliable nanoanalytics, which currently looks like being a problem by its own. Another current major issue is the exposure assessment of humans when confronted with nanomaterials unintentionally released from products or occurring at the workplace and in the environment. All these kinds of problems are in principle not really new. If new chemicals or substances are synthesized and introduced to the market, there should be always analytical measures at hand allowing for detection, quantification, and monitoring of these newcomers. So why should this be a problem and a challenge in the case of nanomaterials?

Nanoanalytics

The classical approach to characterize chemicals is to ask for data on identification, composition, purity, dose, or concentration. From an analytical point of view, this process is well established and comparatively straightforward. However, in order to characterize nanomaterials, this becomes far more complicated and pushes the existing analytical instrumentation right to the limits of what seems technically feasible today. To pursue the goal of getting the risk assessment of nanomaterials to a more advanced level, the following essential questions have to be asked:

- What are the properties of nanomaterials used in a specific product (e.g., nanoclay used as filler in polymers or nanosilver used as surface coating)?
- What are the properties and interactions of nanomaterial-furnished products when actually being in use (e.g., interactions of nanoparticles with biological matrices or other chemical substances through diffusion, migration, and abrasion)?
- What are the properties and interactions of nanomaterials during exposure of living organisms including humans (e.g., after oral, dermal, or inhalative uptake of nanomaterials)?

One central requirement on the metrology employed to gather necessary data is to perform *in situ* measurements under the conditions mentioned above with reliable and reproducible methods in place. For all of the three questions raised above, it has to be asked:

- Which physicochemical properties are really relevant?
- Which currently available analytical techniques are capable of measuring these characteristics?
- How is it possible to strategically combine different analytical techniques to obtain a sufficient dataset for subsequent exposure assessments?

What Does the Nanoparticle See?

The OECD Working Party on Nanotechnology (WPN) has been established in March 2007 to advise upon emerging policy issues of science, technology, and innovation related to the responsible development of nanotechnology [72]. In the following, WPN has published the following list of physicochemical properties to fully characterize individual nanomaterials:

Agglomeration/aggregation	Zeta potential (surface charge)
Water solubility/dispersibility	Surface chemistry (where appropriate)
Crystalline phase	Photocatalytic activity
Dustiness	Pour density
Crystallite size	Porosity
Redox potential	Specific surface area
Radical formation potential	Particle size distribution (dry and in media)

(*continued*)

Representative electron microscopy (TEM) picture(s)	Octanol–water partition coefficient (where relevant)

In addition, OECD also provides a list of fate properties:

Dispersion stability in water	Adsorption, desorption
Biotic degradability	Adsorption to soil or sediment
Identification of degradation products	Bioaccumulation potential
Abiotic degradability and fate	Other relevant environmental fate information (if available)
Further testing of degradation product(s) as required	

Although there are several comparable lists [73–75], the discussion which physicochemical properties are essential to fully characterize and describe nanoparticles is not finished yet. On the other hand, research undoubtedly demonstrated that in biological environments, for instance, in the human body or in food matrix, once being introduced nanoparticles are immediately covered by matrix molecules surrounding the particle, and the so-called corona is formed [76]. For some nanoparticles, several constituents of this corona have been identified yet; different proteins and lipids are among them. The configuration and properties of an individual nanoparticle (i.e., chemical composition, shape, size, and coating) decisively influence which biomolecules will be bound at its surface [77, 78]. Although some biomolecules will be bound quite strongly, forming the so-called "hard corona", others are only loosely bound, and—as a consequence—the biological identity of the particle rather underlies certain dynamics due to its constantly changing "soft corona" at the surface. Through nanoparticle's corona, the surface area, surface reactivity, and surface charge are characterized, thus determining its biological impact and fate [79].

The evolving and changing composition of nanoparticle's corona interacts with the biological material at the molecular level, thereby determining the impact and potential toxicity of the individual particle on its surrounding environment [80]. A closer look reveals that the interface between the nanoparticle and its surrounding medium is shaped by the physicochemical configuration of the material, the solid–liquid interface covering the particle, and the contact zone of the interface interacting with the biological substrate [70, 81]. Although research is mostly concentrated on the interactions of nanoparticles with proteins, a wide range of other biomolecules, like carbohydrates, amino acids, and lipids, can contribute to the composition of the nano-bio interface and thus influence the functionality and signaling of those cells affected (Fig. 4). Based on this notion, the precise characterization of nanoparticles in their particular environment, for example, blood, interstitial fluid, or food, may be one of the key elements crucial for any classification of nanoparticle-mediated risks and the prediction of possible health impacts [82, 83]. However, for industrial relevant nanomaterials, hardly anything is known about the composition of their corona [84, 85]. Furthermore, there is a great lack of systematic data that would be actually necessary to understand how the corona will change in its composition and occurrence dependent on nanoparticle properties. In principle, this situation does not only apply to nanoparticle's protein corona.

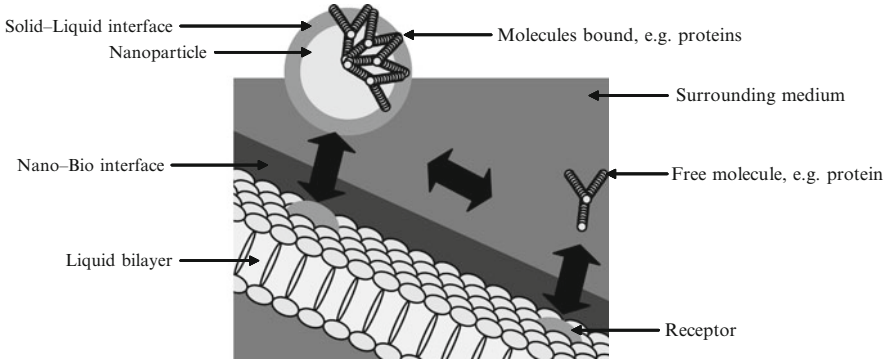


Fig. 4 Interactions at the nano-bio interface: molecules present in the extracellular matrix get adsorbed at nanoparticle's surface and then can bind to the cell membrane, for example, to receptors, just like completely free molecules dissolved in the medium (modified according to a graphic of the Center for Functional Nanostructures (CFN) at KIT, Germany; website: http://www.cfn.kit.edu/)

Rather, it is now well accepted that many physicochemical properties will change over the life cycle of nanomaterials [86, 87]. In conclusion, besides proper characterization of the material right after synthesis, we also need reliable and well-advanced *in situ* analytics of nanomaterials in all kinds of matrices. Research in this direction is only just about to emerge [88].

Measuring Nanoparticles

In analytics, it is common to distinguish between qualitative (e.g., determination of properties) and quantitative (e.g., determination of amounts) assessments. However, for nanomaterials, things reveal more complicated. For instance, determination of the size of a nanoparticle, which obviously looks like a qualitative measure, turns out to be a quantitative measure as well. As the sizes of nanoparticles in a particular preparation follow an individual distribution function, actually, the particle numbers in each size fraction have to be quantified [89].

As it is true in analytics in general, each measurement technique comes with its own limitations, but even more so, in the area of nanomaterials current methods usually offer no more than an estimation. For instance, in dynamic light scattering (DLS) measurements—a technique used to determine the size distribution of particles—readouts are size-weighted as the larger particles contribute much stronger to the scattered signals. In addition, what is actually measured is not the "size" but the hydrodynamic radius, which might result in significant differences for certain nanoparticles [90]. Nevertheless, generally there has been a rapid development for all kinds of analytical techniques in the nanotechnology field. Currently, more than 50 different techniques are used for physicochemical characterization of

nanomaterials. This range of methods encompasses all major areas of analytical chemistry [91, 92], and it would reach far beyond the scope of this introductory chapter to describe them all in more detail. Microscopy, spectroscopy, spectrometry, and separation techniques account for the main part of the analytical portfolio that enables to identify, to separate, and to visualize nanoparticles (Table 3). However, these techniques are thus far only validated for the characterization of nanomaterials under controlled conditions such as, for example, dispersed in water or other defined media or simply in its solid states [93]. Rarely, these methods have yet been tested for nanoparticles embedded or dispersed in complex environments such as human blood and tissues, food, or sewage.

In situ analytics of nanomaterials are meant to also consider and characterize the presence of the aforementioned particle corona and any changes in the aggregation behavior of the particles while being dispersed in biological media. Naturally the constraints of measuring nanoparticles in their particular environmental matrix raise the bar with respect to technological requirements and again lead to the question: Which specific parameters are actually of real importance for characterization in the given context and how to achieve this goal with existing technologies? Ideally from a risk assessment point of few, having the analytical challenges in

Table 3 Overview of currently applied analytical methods in the field of nanomaterials

Category	Technique		Sensitivity	Experience/ distribution based on applications	Costs	Parameters analyzed
Imaging techniques	TEM		h	h	h	Structure
	SEM		h	h	h	Structure
	AFM		m	h	h	Structure
Separation techniques	Chromatography (e.g., HPLC)		m	h	l	Size/structure
	AF4		m	l	l	Size
	CE		l	m	l	Size/charge
Characterization techniques	NMR		l	h	h	Composition/structure
	AU		m	m	l	Size/shape/structure
	MS	ICP	h	h	m / h	Mass/composition
		IM	m	m	m	Mass/composition
		ESI	h	h	m	Mass/composition
		MALDI	h	h	h	Mass/composition
		SIMS	h	l	h	Mass/composition
		DESI	h	l	h	Mass/composition
	DLS		m	m	m	Size/distribution
	SAXS		m	l	h	Size/shape/structure

l low, *m* middle, *h* high, *TEM* transmission electron microscopy, *SEM* scanning electron microscopy, *AFM* atomic force microscopy, *HPLC* high-performance(high-pressure) liquid chromatography, *AF4* asymmetric flow field-flow fractionation, *CE* capillary electrophoresis, *NMR* nuclear magnetic resonance (spectroscopy), *AU* analytical ultracentrifugation, *MS* mass spectrometry, *ICP* inductively coupled plasma, *IM* ion mobility, *ESI* electrospray ionization, *MALDI* matrix-assisted laser desorption/ionization, *SIMS* secondary ion mass spectrometry, *DESI* desorption electrospray ionization, *DLS* dynamic light scattering, *SAXS* small-angle X-ray scattering

mind, these techniques should be well established (validated), robust, and highly selective and sensitive enough for each given analyte.

Nanoparticles: Any Health Risks to be Expected?

To reliably identify the hazards and to assess the potential risks of nanoparticles for humans, we have to consider the following two issues already mentioned above: First, nanoparticles might significantly change the bioavailability of other potentially toxic compounds. In the environment, contaminants are often adhered to solid matrices, one of which could also be represented by the surface of nanoparticles. The efficient binding of chemicals is well documented for many nanomaterials, and this property can be used in a beneficial way to clean, for example, water or soil. CNTs can bind many organic compounds [94] or metals like copper [95] and cobalt [68], zero-valent iron oxide nanoparticles have been shown to adsorb a variety of compounds [96], and a similar behavior is known or expected for other types of nanomaterials as well. As a consequence, potentially toxic compounds might become more bioavailable through facilitated uptake into organisms and subsequent distribution throughout the body, thereby even better penetrating blood–tissue barriers. However, only few studies on this issue (typically in the context of air pollution) were conducted so far. For instance, it could be demonstrated that $FeCl_3$ strongly increases lung inflammation caused by 14-nm carbon black particles *in vivo* [97]. In an *in vitro* study, it was shown that $ZnCl_2$ potentiates TNF-α release in macrophages upon exposure to nanoparticulate air samples [98]. Similarly, putative allergens might attach to the surface of nanoparticles, thereby enhancing allergic reactions. This had been demonstrated for general airborne pollutant particles (diesel exhaust) first and recently also been shown for engineered nanoparticles [99, 100]. Resulting from such observations, nanoparticles are currently also tested as adjuvants in vaccine development [101].

Some of the observed adverse effects might actually result from the scavenging of essential nutrients or endogenous messengers such as hormones, meaning that some nanoparticles could be able to extract physiological compounds from the blood and thus causing a critical shortage in the whole body or in single organs/tissues [102]. Biomolecules that get incorporated into nanoparticle's corona might also undergo changes in their conformation and activity, which in turn can trigger adverse reactions in the whole organism such as, for instance, aberrant plasmatic coagulation or platelet aggregation [103, 104]. However, research in this direction is still extremely limited.

Secondly, the toxic potential of the material itself inevitably is to be evaluated faithfully and comprehensively. However, also in this field, there are usually still extremely limited—if any—data available, and sometimes, the results of different studies may be even contradictory and inconsistent and thus without any value for the assessment of risks [105]. For risk assessment purposes, low-dose, chronic *in vivo* studies would be of highest value as the outcome of such studies can be

used to predict possible overall adverse effects in humans. However, up to now, only for a limited number of nanomaterials (e.g., titanium dioxide, silver, carbon black, CNTs), *in vivo* data exist that typically were obtained through acute or subchronic dosing. Conversely, a large number of *in vitro* studies on nanoparticle toxicity have been published, yet most of them are only of limited value in risk assessment. Since usually extremely high doses are applied as bolus in the *in vitro* systems used, the extrapolating of such data to predict the corresponding outcome in humans is prone to fail. Moreover, the results obtained *in vitro* frequently even reveal inconsistent as recently been summarized for the endpoint genotoxicity [106]. Usually it is hard to figure out whether the inconsistencies observed in nanotoxicological studies are due to improper characterization of the material itself (e.g., unequivocal identity of the particles applied?) or due to an inadequate or flawed design of the study conducted (e.g., proper cell model and suitable endpoint selected?). Overall, the design of studies aimed at assessing nanomaterial toxicity requires much attention to many details as explained nicely in a number of articles [69, 70, 105, 107]. By all means, prior to testing, the nanomaterial needs to undergo copious characterization by several complementary techniques, and the doses finally applied in the experiment should reflect realistic dimensions expected to occur under real-life exposure conditions [70, 105].

In light of the large number of nanomaterials already known and expected to be produced in near future, a tiered testing approach has been proposed comprising a range of different *in vivo* regimes, but also cell-free and cell-based *in vitro* methods [107]. Nevertheless, until today, there is still no final consensus on which validated toxicological assays are appropriate and meaningful enough in the case of nanoparticles, how they optionally have to be adopted to this material, or whether new assays have to be developed and validated instead [69, 107, 108]. Of course, there is great international effort to harmonize national proposals and programs, for example, in the OECD Working Party on Manufactured Nanomaterials (WPMN) [108]. As already mentioned above, only little or even nothing is known for chronic, repeated, and low-dose exposures. Moreover, existing *in vivo* data only cover a small number of organisms, which is especially problematic for ecotoxicological assessments [109]. Mostly these studies employ a small range of established invertebrate models (e.g., *Daphnia magna*) and occasionally certain fish models (e.g., *Danio rerio*). However, virtually no studies exist on plants or other terrestrial organisms [109]. Another limitation is that only a few studies employ more than one organism such that comparison would be feasible, as it was done, for example, by Oberdörster and coworkers [110].

An increasing production and widespread use of nanoparticles enhances the probability of considerable human exposure. Humans can be exposed at workplaces, as consumers or patients or via the environment. Therefore, serious public concern has been raised in recent times about the safety of these materials for humans and the environment [67, 111]. As outlined above, the toxicity of nanoparticles in humans and their environmental fate in good parts result from intrinsic properties of the material and will be influenced by certain physicochemical properties such as size, shape, solubility, or biopersistence (Table 4). To some extent,

Table 4 Selection of physicochemical properties of nanoparticles and kinds of biological responses that are likely to be affected

Composition	Inherent toxicity of the nanomaterial (e.g., nickel, cadmium)
Size	Uptake, translocation, elimination
Shape	Uptake, clearance (e.g., fiber toxicity)
Surface modification	Uptake, interaction with biomolecules
Charge	Uptake, interaction with biomolecules
Conductibility	Interference with transport processes or signaling
Surface area	Interaction with and binding of biomolecules
Solubility	Release of potentially toxic ions, translocation, elimination
Strength/biopersistance	Clearance

these properties are expected to change depending on the particular biological environment.

The most important exposure routes of humans to be considered for nanoparticles are skin penetration, ingestion, and inhalation [70]. The skin represents an organ with large surface (about 1.5–2 m^2 for adults), and its architecture reveals kind of complex due to stratification. So, the outermost part is made by the stratum corneum barrier, which consists of dead cells and which is followed by the living layers in the epidermis and dermis. The epidermis does not contain blood vessels but is composed of keratinocytes, melanocytes, Langerhans cells, and Merckel cells. The basement membrane then connects the epidermis with the dermis beneath, which contains blood vessels, neurons, and hair roots and which is made of fibroblasts, adipose cells, macrophages, and dendritic cells. Since nanoparticles are also applied in various cosmetic products, dermal exposure and dermal penetration have been extensively studied in several *in vivo* and *in vitro* studies [112, 113]. Most *in vivo* studies have been performed with titanium dioxide (TiO_2) and zinc oxide (ZnO), which is reasonable as these metal oxides are currently widely applied in nanoparticulate configuration in sunscreens, typically with particle sizes of 20–50 nm. So far, there is no evidence that the stratum corneum barrier can be penetrated by such particles so that viable tissue will be reached [114–116]. Sometimes, it was observed that the nanoparticles entered hair follicles, and from this, it was concluded that the hair follicles might act as a reservoir that could facilitate dermal penetration [117]. However, since conclusive data were not presented, there is currently wide agreement that these particles tested do not reach viable cell layers in the skin, neither directly nor via hair follicles. Based on this, there was broad consensus that the dermal route is to be considered safe for nanoparticulate TiO_2 [118]. However, the range of nanoparticle types studied with regard to their dermal uptake is still very low, and—as noted above—the field was mainly concentrated on metal oxides. By contrast, investigation of other types of nanoparticles such as silver provided evidence that it well might be possible that such species penetrate into living skin [119]. Similar results were observed for fluorescent particles (fluorospheres) of 0.5 and 1 μm diameter that could reach viable epidermal and dermal layers [120]. It also should be taken into account that all of these data refer to intact and undamaged skin tissue. By contrast, damaged skin (e.g., UV burned) or skin from susceptible individuals afflicted by

skin diseases such as atopic eczema (neurodermitis) usually is not been considered. One study with hairless mice demonstrated that susceptible skin might be penetrated by nanoparticles quite easily [121]. This clearly shows that further work is needed also for the dermal route.

The second exposure route, that is, the oral or ingestion route, is only poorly addressed so far [122]. Most of the published studies are related to nanomedicine and drug delivery issues. It has been shown that microfold or M cells of the Peyer's patches in the small intestine, which can ingest particulate matter, might represent the major route of intestinal uptake of nanoparticles [122]. One study with nanogold demonstrated a size dependency of the uptake in the small intestine [123]. Investigation of 56-nm silver particles in a subchronic study revealed gastrointestinal absorption and subsequent systemic distribution toward a wide range of different organs including bladder, heart, lungs, prostate, kidneys, spleen, liver, brain, *etc*. [124]. In addition, signs of liver toxicity at medium and high doses of nanosilver were observed in this study. Other studies were performed with polystyrene or latex particles. Recently, one study showed that upon oral administration, 300 nm poly-D,L-lactide-co-glycolide (PLGA) particles could be detected in liver, kidney, brain, and other organs [125]. In general, however, the number of available *in vivo* and *in vitro* studies addressing the intestinal absorption of nanoparticles is as yet not sufficient enough to draw any health-related conclusions from it.

The inhalation route has gained great attention in recent years, and in fact, most *in vivo* studies in the field of nanotoxicology published so far relate to inhalation exposure [70]. It is considered the most relevant exposure route especially for workplace exposures [70]. Furthermore, a significant hazard was expected based on experiences with asbestos or crystalline silica (i.e., quartz, cristobalite), both of which are among classified carcinogens [126, 127]. Also, many experiences exist from studies with ambient air particulate matter (PM), which is traditionally classified according to its respective size as PM_{10}, $PM_{2.5}$, and $PM_{0.1}$ (see chapter on *Toxicology of Ambient Particulate Matter*, authored by van Berlo *et al*.). In terms of size, the $PM_{0.1}$ fraction would pertain to nanoparticles as it covers particulate matter in a size range below 100 nm. For ambient particulate matter, it was deduced that it is mainly the ultrafine fraction ($PM_{0.1}$) within many kinds of (environmental) pollution which causes adverse effects such as cardiovascular dysfunction [128].

Large numbers of studies exist on the size-dependent deposition and clearing of particles in the lungs [70, 129]. The upper airway and lower respiratory tract, down to the bronchial tubes (bronchi) and bronchioles, are covered by an epithelium and a small lining of tiny cilia which act as filter and motor to move the epithelial mucus upward. So, the main clearance mechanism in this ciliated region of the respiratory tract enables to trap the particles in the mucus and to subsequently push them upward toward the trachea via the so-called mucociliary escalator mechanism. By contrast, the most proximal endings of the tracheobronchial tree, that is, the gas-exchanging alveoli, are neither covered with cilia nor possess any mucus layer; instead, they produce surfactant [129]. Particle clearance in this part of the lungs is only feasible via cellular phagocytosis, mainly executed by pulmonary (i.e., alveolar) macrophages or by dissolution and subsequent removal via bloodstream.

Particles below 2.5 μm can reach even these lowest parts of the pulmonary tract and are being potentially eliminated via both kinds of mechanisms [70]. In this context, it should be mentioned that the International Commission of Radiological Protection (ICRP) proposed a prediction model which also enables for estimation of the amounts of particles deposited in each lung compartment depending on the particle size [130]. According to this, more than 90% of very small nanoparticles (1 nm) retain in the nasopharyngeal region, while the remaining fraction distributes in the tracheobronchial tract without reaching the alveoli. By contrast, about 50% of 20-nm particles would spread far down and populate the pulmonary alveoli [70].

It has been proposed that the higher inhalation toxicity of nanoparticles compared to their corresponding bulk particles not solely results from the facilitated and deeper penetration of the smaller particles into the region of pulmonary alveoli. To prove this, Oberdörster and coworkers used TiO_2 particles of two different primary sizes (25 nm and 250 nm) but with the same overall hydrodynamic radius (1 μm), thus leading to the assumption that the deposition behavior in the lungs most likely would be comparable for both. The results of the studies performed, however, showed that the 250-nm particles failed to induce pulmonary inflammation, while the 25-nm particles did [131, 132]. Similar studies have been performed with other types of nanoparticles such as carbon black [133, 134] or polystyrene beads [135]. The current conclusion drawn from the data obtained is that the increased surface area of nanoparticles, compared to corresponding bulk particles, which is also partially retained in agglomerates, will cause oxidative stress and inflammation in the distal pulmonary tract. An additional major concern derived from these *in vivo* studies comes from the observed translocation of particles to extrapulmonary tissues [70, 136]. It is thus highly likely that nanoparticles may reach secondary target organs through the pulmonary exposure route. These secondary organs and tissues can then be adversely affected, for instance, the cardiovascular system, the spleen, or the brain [137–139].

Due to time constraints, monetary aspects, and—first and foremost—animal welfare considerations, for sure it is inconceivable to characterize all kinds of different nanoparticles and nanomaterials regarding their safety and toxicological behavior via long-term *in vivo* studies. Instead, robust and reliable non-animal testing approaches based on *in vitro* and *in silico* methods need to be developed. As another objection against any full-range *in vivo* testing philosophy, the knowledge gained through whole animal studies into the *molecular mechanisms* that may underlie any kind of nanotoxicological effect and its transferability to the human system is likely to be limited. To date, a large amount of *in vitro* studies are published, which all report some adverse effects—at least in high concentration ranges—of a large variety of different nanoparticles (cf. above). For instance, the available data for titanium dioxide, nanosilver, and nanogold are nicely summarized in several reviews [140–142]. Most of the *in vitro* studies report on oxidative stress, cytotoxicity, and inflammatory responses. For several types of nanoparticles, it has been shown in a range of different cell lines that reactive oxygen species (ROS) are being formed and/or the levels of glutathione become depleted [143]. In addition, it could be demonstrated that certain signaling pathways like NFκB or

AP-1 are being activated [144]. In terms of genotoxicity, the data are still contradictory and inconsistent [106]. Currently, there is much discussion about how to harmonize such studies, how intense the characterization needs to be done in advance, and which readouts are suitable and meaningful enough [107, 108]. Finally, besides harmonization, validation is necessary as well. At OECD WPMN [108], one working package is commissioned to particularly focus on the further development of *in vitro* assays. Beyond that there is intense discussion on how to correlate data obtained *in vitro* to the *in vivo* situation expected to be present in all kinds of organs and tissues in the body [145, 146]. A general problem is the issue of dosimetry. Traditionally also nanoparticle dosages are reported as mass doses (e.g., mg/L). However, there is evidence that other dose metrics, such as particle number or surface, might be better suited and more meaningful [70]. Although it seems that—to a good part—data might be calculationally converted from one dose metric to another, there can be severe limitations as the shape or the agglomeration grade might vary from batch to batch or from study to study. So, it still looks that several unresolved issues pave the road toward reliable and resilient risk assessment approaches required for safe nanotechnology applications in the years ahead.

Perspectives

To further develop nanotoxicological assays, we need to better understand which kinds of physicochemical properties of the particles may exert an influence on the overall adversity in cells and tissues and by which mechanisms. This set of characteristics is likely to encompass intrinsic material properties such as size or shape, but also dynamic properties, such as the corona, that may change over the life cycle of particles. Currently existing data gaps are huge, which does not really come as surprise given that nanotechnology is still a young and heavily developing branch of science. These data gaps pertain to both core elements of any risk assessment, that is, the quantitative characterization of external and internal exposures as well as the dose dependency of toxicological effects [147, 148]. As for exposure assessments, there is still the issue awaiting to be resolved how to measure nanoparticles in their natural environments. High background levels from natural (nano)particles exist and, thus, currently available techniques usually reach their limits. Ideally a measurement method would be able to quantitatively assess the number of particles in a certain size range and—at the same time—provide information about their chemical composition. Since such analytical instrumentation is currently unavailable, at the present state we can only obtain estimates on the levels of exposures in the general environment or under consumer conditions. On the other hand, this situation might be different for workplaces, where the type of material and the putative emission source are usually known. Therefore, currently serious strategies for the assessment of exposures to nanoparticles only exist for occupational environments [149].

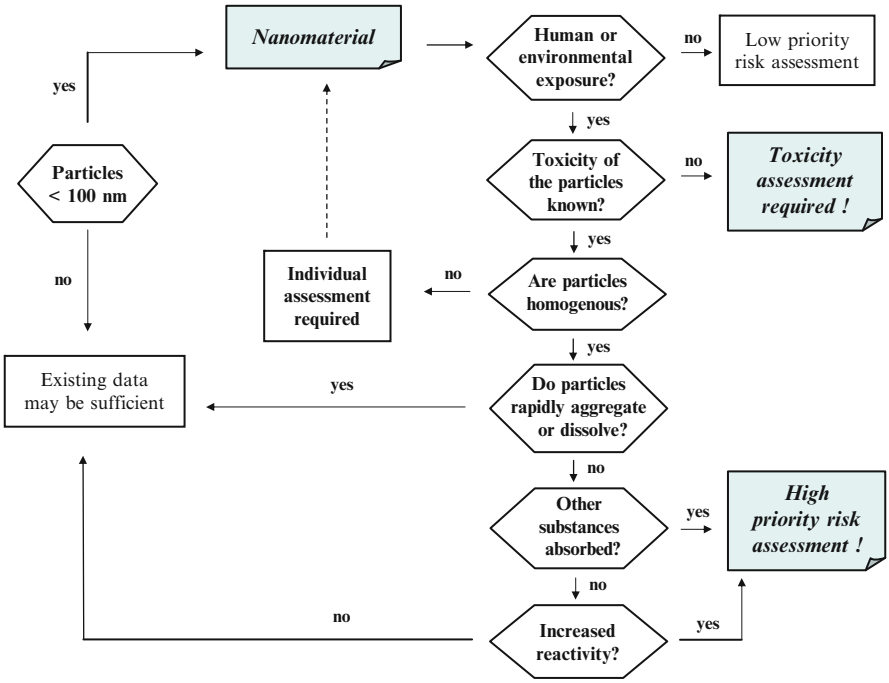


Fig. 5 Flowchart of a possible risk assessment decision tree applicable for nanoparticles and nanomaterials (adapted from [150])

Important for the toxicological assessment of nanomaterials, uncertainties also exist on which and how abiotic factors might contribute to the time-dependent alteration of nanoparticle's properties and thus how they would influence its toxicity. We almost know nothing about how mixtures or formulations may contribute or alter inherent properties and the environmental behavior of nanoparticles. As it is not even conceivable to introduce each different type of nanomaterial into a conventional testing strategy, it would be completely beyond any scope to test all kinds of possible mixtures or formulations individually. Hence, we have to find alternative ways to deal with and to successfully address these issues. Another major lack of knowledge relates to low and repeated dose exposures and to chronic endpoints. Given all of this, it is obvious that significant work needs to be done before regulators will be in a more comfortable situation with regard to health safety considerations.

Several authors made suggestions how currently a decision tree for risk assessment of nanomaterials could look like. An example is given in Fig. 5.

Risk assessment of chemicals in general and of nanoparticles or nanomaterials in particular should be performed in an evidence-based, robust, and transparent way, and the final conclusions drawn need to be comprehensive, reasonable, and logic. As explained in detail above, the evidence base for nanomaterials is still extremely holey and incomplete. It seems reasonable that new technologies may need some

time until all methods required for characterization and testing are sufficiently developed and in place. In the field of nanotechnology, however, the closing of data gaps will be crucial for the development of risk assessment strategies and for establishing regulatory measures [151]. Moreover, it even will be key for no less than the further development and the general acceptance of this technology in the public. To reach this goal, we need to be as soon as possible in a situation where all of the following questions can be answered by a "yes":

- Are the existing methods of testing (exposure and toxicology) sufficiently suited for nanomaterials?
- Are our risk assessment strategies reliably applicable and sufficiently suited for nanomaterials?
- Is our current legislation sufficient to cover also nanomaterials and all application fields adequately?

Certainly, there is still a long way to go in the safety field pertaining to nanotechnology. The past years have taught us, however, that thanks to worldwide efforts we can gain much progress even in such complicated areas in short term. In light of the most current developments in analytical instrumentation, exposure monitoring measures, and non-animal testing procedures, now, much more than 5 or 10 years ago, it becomes conceivable to reach a point in the future where the continuing evolution of nanotechnology can be accompanied, backed up, and supported by adequate safety assessments of and decision-making on the materials produced [151].

References

1. Oberdörster G (2010) Nanotoxicology: need for a science based risk assessment. NanoSAFE 2010, Grenoble, France. Online available at http://www.nanosafe.org/home/liblocal/docs/Nanosafe%202010/2010_oral%20presentations/PL4_Oberdorster.pdf
2. Schummer J, Baird D (2006) Nanotechnology challenges: implications for philosophy, ethics and society. World Scientific Publishing, Singapore
3. NSTC (1999) Nanotechnology research directions: IWGN Workshop Report. Vision for Nanotechnology R&D in the Next Decade. National Science and Technology Council (NSTC), Committee on Technology (CT), Interagency Working Group on Nanoscience, Engineering and Technology (IWGN), MC Roco, RS Williams, P Alivisatos (eds), Washington DC. Online available at http://www.wtec.org/loyola/nano/IWGN.Research.Directions/IWGN_rd.pdf
4. NSTC (2000) Nanotechnology research directions for societal needs in 2020. Retrospective and outlook summary. In: Roco MC, Mirkin CA, Hersam MC (eds) Science Policy Reports, WTEC. Springer, Dordrecht, The Netherlands. Online available at http://www.wtec.org/nano2/docs/Nano2-Brochure-Final-04-14-11.pdf
5. Baalousha M, Lead JR (2009) Overview of nanoscience in the environment. In: Lead JR, Smith E (eds) Environmental and human health impacts of nanotechnology. Wiley-VCH, Chichester, pp 1–29
6. Walter P, Welcomme E, Hallégot P, Zaluzec NJ, Deeb C, Castaing J, Veyssière P, Bréniaux R, Lévêgue JL, Tsoucaris G (2006) Early use of PbS nanotechnology for an ancient hair dyeing formula. Nano Lett 6:2215–2219

7. Currall SC, King EB, Lane N, Madera J, Turner S (2006) What drives public acceptance of nanotechnology? Nat Nanotechnol 1:153–155
8. Rotello VM (2004) Nanoparticles: building blocks for nanotechnology. Springer, Heidelberg, Germany
9. Monteiro-Riviere NA, Tran CL (2007) Nanotoxicology: characterization, dosing, and health effects. Informa Healthcare, New York, NY
10. Sahu SC, Casciano DA (2009) Nanotoxicity: from *in vivo* and *in vitro* models to health risks. Wiley-VCH, Chichester
11. Lead JR, Smith E (2009) Environmental and human health impacts of nanotechnology. Wiley-VCH, Chichester
12. Murashov V, Howard J (2011) Nanotechnology standards. Springer, New York, NY
13. Chen PC, Mwakwari SC, Oyelere AK (2008) Gold nanoparticles: from nanomedicine to nanosensing. Nanotech Sci Appl 1:45–66
14. Kunckel J (1679) Ars Vitraria Experimentalis Oder vollkommene Glasmacher-Kunst. Frankfurt-Leipzig, Germany
15. Faraday M (1857) The Bakerian lecture: experimental relations of gold (and other metals) to light. Philos Trans R Soc Lond 147:145–181
16. Schramm LL (2008) Dictionary of nanotechnology. Colloid and Interface Science, Wiley-VCH, Weinheim, Germany
17. Feynman RP (1960) There's plenty of room at the bottom. Caltech Eng Sci 23:22–26
18. Binning G, Rohrer H, Gerber C, Weibel E (1982) Surface studies by scanning tunneling microscopy. Phys Rev Lett 49:57–61
19. Binning G, Quate CF, Gerber C (1986) Atomic force microscope. Phys Rev Lett 56:930–933
20. Taniguchi N (1974) On the basic concept of nanotechnology. In: Proceedings of the international conference on production engineering, Tokyo, Part II, Japan Society of Precision Engineering. Japan Society of Precision Engineering, Tokyo
21. Kreuter J (1983) Evaluation of nanoparticles as drug-delivery system. I. Preparation methods. Pharm Acta Helv 58:196–209
22. Lehtipalo K, Kulmala M, Sipilä M, Petäjä T, Vana M, Ceburnis D, Dupuy R, O'Dowd C (2010) Nanoparticles in boreal forest and coastal environment: a comparison of observations and implications of the nucleation mechanism. Atmos Chem Phys 10:7009–7016
23. Bertsch PM, Seaman JC (1999) Characterization of complex mineral assemblages: implications for contaminant transport and environment remediation. Proc Natl Acad Sci USA 96:3350–3357
24. Williams PM, Druffel ERM (1988) Dissolved organic matter in the ocean: comments on a controversy. Oceanography 1:14–17
25. Borm P, Klaessig FC, Landry TD, Moudgil B, Pauluhn J, Thomas K, Trottier R, Wood S (2006) Research strategies for safety evaluation of nanomaterials. Part V: role of dissolution in biological fate and effects of nanoscale particles. Toxicol Sci 90:23–32
26. NNI (2004) U.S. National Nanotechnology Initiative. Online available at http://www.nano.gov/
27. Shenderova OA, Zhirnov VV, Brenner DW (2002) Carbon nanostructures. Crit Rev Sol Stat Mat Sci 27:227–356
28. Hodge GA, Bowman DM, Maynard AD (2010) International Handbook on Regulating Nanotechnologies. Edward Elgar, Cheltenham, UK. Online available at http://umrscblogs.org/wp-content/uploads/2011/02/Handbook-Regulating_nanotech_ch126.pdf
29. CEN/ISO (2008) Nanotechnologies: terminology and definitions for nano-objects—nanoparticle, nanofibre and nanoplate. European Committee for Standarizaton, Technical Specification (TS) 27687, DIN SPEC, Deutsches Institut für Industrienormung e.V., Berlin, Germany
30. Bernard D (2010) Nanomaterials and uncertainties: regulation needs and standardisation answers. NanoSAFE 2010, Grenoble, France. Online available at http://www.nanosafe.org/home/liblocal/docs/Nanosafe%202010/2010_oral%20presentations/PL9_Bernard.pdf

31. Maynard A, Bowman D, Hodge G (2011) The problem of regulating sophisticated materials. Nat Mater 10:554–557
32. BSI (2005) Vocabulary. Nanoparticles. PAS 71:2005, British Standards Institution, BSI Corporate, London, UK. Online available at http://shop.bsigroup.com/en/ProductDetail/?pid=000000000030127836
33. BSI (2007) Terminology for nanomaterials. PAS 136:2007, British Standards Institution, BSI Corporate, London, UK. Online available at http://shop.bsigroup.com/Navigate-by/PAS/
34. Cuenya BR (2010) Synthesis and catalytic properties of metal nanoparticles: size, shape, support, composition, and oxidation state effects. Thin Solid Films 518:3127–3150
35. Kelly KL, Coronado E, Zhao LL, Schatz GC (2003) The optical properties of metal nanoparticles: the influence of size, shape, and dielectric environment. J Phys Chem 107:668–677
36. Bhattacharya R, Mukherjee P (2008) Biological properties of "naked" metal nanoparticles. Adv Drug Deliv Rev 60:1289–1306
37. Christian P (2009) Nanomaterials: properties, preparation and applications. In: Lead JR, Smith E (eds) Environmental and human health impacts of nanotechnology. Wiley, Chichester, pp 31–77
38. Bukowski T, Simmons J (2002) Quantum dot research: current state and future prospects. Crit Rev Solid Stat Mat Sci 27:119–142
39. Lu X, Rycenga M, Skrabalak SE, Wiley B, Xia Y (2009) Chemical synthesis of novel plasmonic nanoparticles. Annu Rev Phys Chem 60:167–192
40. Abrams BL, Wilcoxon JP (2005) Nanosize semiconductors for photooxidation. Crit Rev Solid Stat Mat Sci 30:153–182
41. Milliron DJ, Alivisatos AP, Pitois C, Edder C, Fréchet JMJ (2003) Electroactive surfactant designed to mediate electron transfer between CdSe nanocrystals and organic semiconductors. Adv Mat 15:58–61
42. Zhao CM, Wang WX (2011) Importance of surface coatings and soluble silver in silver nanoparticles toxicity to *Daphnia magna*. Nanotoxicology (Epub ahead)
43. Roco MC (2005) International perspective on government nanotechnology funding in 2005. J Nanopart Res 7:707–712
44. Park K (2007) Nanotechnology: what it can do for drug delivery. J Control Release 120:1–3
45. Ketchie W, Fang Y, Wong M, Murayama M, Davis R (2007) Influence of gold particle size on the aqueous-phase oxidation of carbon monoxide and glycerol. J Catal 250:94–101
46. Panigrahi S, Basu S, Praharaj S, Pande S, Jana S, Pal A, Ghosh SK, Pal T (2007) Synthesis and size-selective catalysis by supported gold nanoparticles: study on heterogeneous and homogeneous catalytic process. J Phys Chem C 111:4596–4605
47. Levine S (1939) Problems of stability in hydrophobic colloidal solutions. I. On the interaction of two colloidal metallic particles. General discussion and applications. Proc R Soc Lond 170:145–164
48. Aveyard R, Binks BP, Clint JH (2003) Emulsions stabilised solely by colloidal particles. Adv Colloid Interface Sci 100–102:503–546
49. Resch-Genger U, Grabolle M, Cavaliere-Jaricot S, Nitschke R, Nann T (2008) Quantum dots *versus* organic dyes as fluorescent labels. Nat Methods 5:763–775
50. Blanco E, Hsiao A, Mann AP, Landry MG, Meric-Bernstam F, Ferrari M (2011) Nanomedicine in cancer therapy: innovative trends and prospects. Cancer Sci 102:1247–1252
51. Yih TC, Al-Fandi M (2006) Engineered nanoparticles as precise drug delivery systems. J Cell Biochem 97:1184–1190
52. Petrelli F, Borgonovo K, Barni S (2010) Targeted delivery for breast cancer therapy: the history of nanoparticle-albumin-bound paclitaxel. Exp Opin Pharmacother 11:1413–1432
53. Mansour HM, Rhee YS, Wu X (2009) Nanomedicine in pulmonary delivery. Int J Nanomedicine 4:299–319
54. Costantino L (2010) Drug delivery to the CNS and polymeric nanoparticulate carriers. Future Med Chem 2:1681–1701

55. Woodrow Wilson International Center for Scholars. The Project on Emerging Nanotechnologies (PEN). Woodrow Wilson Database on Consumer Products. Online available at http://www.nanotechproject.org/inventories/consumer/
56. Nohynek GJ, Antignac E, Re T, Toutain H (2010) Safety assessment of personal care products/cosmetics and their ingredients. Toxicol Appl Pharmacol 243:239–259
57. Wiechers JW, Musee N (2010) Engineered inorganic nanoparticles and cosmetics: facts, issues, knowledge gaps and challenges. J Biomed Nanotechnol 6:408–431
58. Ross BL, Aitken RJ (2010) Technology Sector Evaluation: Textiles. European Nanotechnology Gateway. ObservatoryNANO. Online available at http://www.observatorynano.eu/project/
59. Upadhyayula VK, Deng S, Mitchell MC, Smith GB (2009) Application of carbon nanotube technology for removal of contaminants in drinking water: a review. Sci Total Environ 408:1–13
60. Kwon S, Fan M, Cooper AT, Yang H (2008) Photocatalytic applications of micro- and nano-TiO_2 in environmental engineering. Crit Rev Environ Sci Technol 38:197–226
61. Kanel SR, Greneche JM, Choi H (2006) Arsenic(V) removal from groundwater using nano scale zero-valent iron as a colloidal reactive barrier material. Environ Sci Technol 40:2045–2050
62. Diallo MS, Christie S, Swaminathan P, Balogh L, Shi X, Um W, Papelis C, Goddard WA 3rd, Johnson JH Jr (2004) Dendritic chelating agents. 1. Cu(II) binding to ethylene diamine core poly(amidoamine) dendrimers in aqueous solutions. Langmuir 20:2640–2651
63. Diallo MS, Arasho W, Johnson JH Jr, Goddard WA 3rd (2008) Dendritic chelating agents. 2. U(VI) binding to poly(amidoamine) and poly(propyleneimine) dendrimers in aqueous solutions. Environ Sci Technol 42:1572–1579
64. Barsan N, Schweizer-Berberich M, Göpel W (1999) Fundamental and practical aspects in the design of nanoscaled SnO_2 gas sensors: a status report. Fresenius J Anal Chem 365:287–304
65. Kim YC, Sasaki S, Yano K, Ikebukuro K, Hashimoto K, Karube I (2001) Photocatalytic sensor for the determination of chemical oxygen demand using flow injection analysis. Anal Chim Acta 432:59–66
66. Sinnott SB, Andrews R (2001) Carbon nanotubes: synthesis, properties, and applications. Crit Rev Solid Stat Mat Sci 26:145–249
67. Seaton A, Tran L, Aitken R, Donaldson K (2010) Nanoparticles, human health hazard and regulation. J R Soc Interface 7(Suppl 1):S119–S129
68. Ebbesen TW, Ajayan PM, Hiura H, Tanigaki K (1994) Purification of nanotubes. Nature 367:519
69. Maynard AD, Aitken RJ, Butz T, Colvin V, Donaldson K, Oberdörster G, Philbert MA, Ryan J, Seaton A, Stone V, Tinkle SS, Tran L, Walker NJ, Warheit DB (2006) Safe handling of nanotechnology. Nature 444:267–269
70. Oberdörster G, Oberdörster E, Oberdörster J (2005) Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. Environ Health Perspect 113:823–839
71. Simkó M, Mattsson MO (2010) Risks from accidental exposures to engineered nanoparticles and neurological health effects: a critical review. Part Fibre Toxicol 7:42–57
72. OECD Working Party on Nanotechnology (WPN). Online available at http://www.oecd.org/document/35/0,3746,en_21571361_41212117_42378531_1_1_1_1,00.html
73. Crane M, Handy RD (2007) An assessment of regulatory testing strategies and methods for characterizing the ecotoxicological hazards of nanomaterials. Watts & Crane Associates, Report for Defra, London, UK. Online available at http://randd.defra.gov.uk/Document.aspx?Document=CB01097_6262_FRP.pdf
74. Crane M, Handy RD, Garrod J, Owen R (2008) Ecotoxicity test methods and environmental hazard assessment for engineered nanoparticles. Ecotoxicology 17:421–437
75. Warheit DB, Sayes CM, Reed KL, Swain KA (2008) Health effects related to nanoparticle exposures: environmental, health and safety considerations for assessing hazards and risks. Pharmacol Ther 120:35–42

76. Lundqvist M, Stigler J, Elia G, Lynch I, Cedervall T, Dawson KA (2008) Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts. Proc Natl Acad Sci USA 105:14265–14270
77. Jiang X, Weise S, Hafner M, Röcker C, Zhang F, Parak WJ, Nienhaus GU (2010) Quantitative analysis of the protein corona on FePt nanoparticles formed by transferrin binding. J R Soc Interface 7(Suppl 1):S5–S13
78. Röcker C, Pötzl M, Zhang F, Parak WJ, Nienhaus GU (2009) A quantitative fluorescence study of protein monolayer formation on colloidal nanoparticles. Nat Nanotechnol 4:577–580
79. Cedervall T, Lynch I, Lindman S, Berggård T, Thulin E, Nilsson H, Dawson KA, Linse S (2007) Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles. Proc Natl Acad Sci USA 104:2050–2055
80. Nel AE, Mädler L, Velegol D, Xia T, Hoek EM, Somasundaran P, Klaessig F, Castranova V, Thompson M (2009) Understanding biophysicochemical interactions at the nano-bio interface. Nat Mater 8:543–557
81. Oberdörster G, Maynard A, Donaldson K, Castranova V, Fitzpatrick J, Ausman K, Carter J, Karn B, Kreyling W, Lai D, Olin S, Monteiro-Riviere N, Warheit D, Yang H (2005) Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. Part Fibre Toxicol 2:8
82. Kroll A, Pillukat MH, Hahn D, Schnekenburger J (2009) Current *in vitro* methods in nanoparticle risk assessment: limitations and challenges. Eur J Pharm Biopharm 72:370–377
83. Jones CF, Grainger DW (2009) *In vitro* assessments of nanomaterial toxicity. Adv Drug Deliv Rev 61:438–456
84. Landsiedel R, Ma-Hock L, Kroll A, Hahn D, Schnekenburger J, Wiench K, Wohlleben W (2010) Testing metal-oxide nanomaterials for human safety. Adv Mater 22:2601–2627
85. SCENIHR (2009) Risk assessment of products of nanotechnologies. Scientific committee on emerging and newly identified health risks, European commission, health & consumers DG, Brussels, Belgium. Online available at http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_023.pdf
86. Tiede K, Hassellöv M, Breitbarth E, Chaudhry Q, Boxall AB (2009) Considerations for environmental fate and ecotoxicity testing to support environmental risk assessments for engineered nanoparticles. J Chromatogr A 1216:503–509
87. Stone V, Nowack B, Baun A, van den Brink N, Kammer F, Dusinska M, Handy R, Hankin S, Hassellöv M, Joner E, Fernandes TF (2010) Nanomaterials for environmental studies: classification, reference material issues, and strategies for physico-chemical characterisation. Sci Total Environ 408:1745–1754
88. EFSA (2009) The potential risks arising from nanoscience and nanotechnologies on food and feed safety. Scientific Opinion of the Scientific Committee. EFSA J 958: 1–39. Online available at http://www.efsa.europa.eu/de/scdocs/doc/958.pdf
89. Khlebtsov BN, Khlebtsov NG (2011) On the measurement of gold nanoparticle sizes by the dynamic light scattering method. Colloid J 73:118–127
90. European Nanotechnology Gateway (2006) *Nanometrology*. Nanoforum.org 8th Nanoforum Report. Online available at http://www.nanoforum.org
91. Powers KW, Brown SC, Krishna VB, Wasdo SC, Moudgil BM, Roberts SM (2006) Research strategies for safety evaluation of nanomaterials. Part VI. Characterization of nanoscale particles for toxicological evaluation. Toxicol Sci 90:296–303
92. Powers KW, Palazuelos M, Moudgil BM, Roberts SM (2007) Characterization of the size, shape, and state of dispersion of nanoparticles for toxicological studies. Nanotoxicology 1:42–51
93. Klaine SJ, Alvarez PJ, Batley GE, Fernandes TF, Handy RD, Lyon DY, Mahendra S, McLaughlin MJ, Lead JR (2008) Nanomaterials in the environment: behavior, fate, bioavailability, and effects. Environ Toxicol Chem 27:1825–1851

94. Long RQ, Yang RT (2001) Carbon nanotubes as superior sorbent for dioxin removal. J Am Chem Soc 123:2058–2059
95. Liang P, Ding Q, Song F (2006) Application of multiwalled carbon nanotubes as solid phase extraction sorbent for preconcentration of trace copper in water samples. J Sep Sci 28: 2339–2343
96. Zhang WS (2003) Nanoscale iron particles for environmental remediation: an overview. J Nanopart Res 5:323–332
97. Wilson MR, Lightbody JH, Donaldson K, Sales J, Stone V (2002) Interactions between ultrafine particles and transition metals *in vivo* and *in vitro*. Toxicol Appl Pharmacol 184: 172–179
98. Wilson MR, Foucaud L, Barlow PG, Hutchison GR, Sales J, Simpson RJ, Stone V (2007) Nanoparticle interactions with zinc and iron: implications for toxicology and inflammation. Toxicol Appl Pharmacol 225:80–89
99. Takano H, Ichinose T, Miyabara Y, Shibuya T, Lim HB, Yoshikawa T, Sagai M (1998) Inhalation of diesel exhaust enhances allergen-related eosinophil recruitment and airway hyperresponsiveness in mice. Toxicol Appl Pharmacol 150:328–337
100. Harkema JR, Rowley N, Bramble L, Zhang Q, Jackson-Humbles D, Baker G, Wagner J, Worden M (2011) Engineered silica nanoparticles act as adjuvants to enhance allergic airway disease in mice. Society of Toxicology (SOT), 50th Anniversary Meeting, March 6–10, Washington DC, USA, Abstract #1463
101. Peek LJ, Middaugh CR, Berkland C (2008) Nanotechnology in vaccine delivery. Adv Drug Deliv Rev 60:915–928
102. Horie M, Nishio K, Fujita K, Endoh S, Miyauchi A, Saito Y, Iwahashi H, Yamamoto K, Murayama H, Nakano H, Nanashima N, Niki E, Yoshida Y (2009) Protein adsorption of ultrafine metal oxide and its influence on cytotoxicity toward cultured cells. Chem Res Toxicol 22:543–553
103. Vertegel AA, Siegel RW, Dordick JS (2004) Silica nanoparticle size influences the structure and enzymatic activity of adsorbed lysozyme. Langmuir 20:6800–6807
104. Khandoga A, Stoeger T, Khandoga AG, Bihari P, Karg E, Ettehadieh D, Lakatos S, Fent J, Schulz H, Krombach F (2010) Platelet adhesion and fibrinogen deposition in murine microvessels upon inhalation of nanosized carbon particles. J Thromb Haemost 8:1632–1640
105. Rushton EK, Jiang J, Leonard SS, Eberly S, Castranova V, Biswas P, Elder A, Han X, Gelein R, Finkelstein J, Oberdörster G (2010) Concept of assessing nanoparticle hazards considering nanoparticle dosemetric and chemical/biological response metrics. J Toxicol Environ Health A 73:445–461
106. Landsiedel R, Kapp MD, Schulz M, Wiench K, Oesch F (2009) Genotoxicity investigations on nanomaterials: methods, preparation and characterization of test material, potential artifacts and limitations—many questions, some answers. Mutat Res 681:241–258
107. Warheit DB, Borm PJ, Hennes C, Lademann J (2007) Testing strategies to establish the safety of nanomaterials: conclusions of an ECETOC workshop. Inhal Toxicol 19:631–643
108. OECD Working Party on Manufactured Nanomaterials (WPMN). Website: http://www.oecd.org/document/47/0,3746,en_2649_37015404_41197295_1_1_1_1,00.html
109. Apte SC, Roger NJ, Batley GE (2009) Ecotoxicology of manufactured nanoparticles. In: Lead JR, Smith E (eds) Environmental and human health impacts of nanotechnology. Wiley, Chichester, pp 267–306
110. Oberdörster E, Zhu S, Blickley TM, McClellan-Green HML (2006) Ecotoxicology of carbon-based engineered NPs: effects of fullerene (C_{60}) on aquatic organisms. Carbon 44: 1112–1120
111. Hristozov D, Malsch I (2009) Hazards and risks of engineered nanoparticles for the environment and human health. Sustainability 1:1161–1194
112. Baroli B (2010) Skin absorption and potential toxicity of nanoparticulate nanomaterials. J Biomed Nanotechnol 6:485–496

113. Smijs TG, Bouwstra JA (2010) Focus on skin as a possible port of entry for solid nanoparticles and the toxicological impact. J Biomed Nanotechnol 6:469–484
114. Pflücker F, Hohenberg H, Hölzle E, Will T, Pfeiffer S, Wepf R, Diembeck W, Wenck H, Gers-Barlag H (1999) The outermost stratum corneum layer is an effective barrier against dermal uptake of topically applied microsized titanium dioxide. Int J Cosmet Sci 21:399–411
115. Pflücker F, Wendel V, Hohenberg H, Gärtner E, Will T, Pfeiffer S, Wepf R, Gers-Barlag H (2001) The human stratum corneum layer: an effective barrier against dermal uptake of different forms of topically applied microsized titanium dioxide. Skin Pharmacol Appl Skin Physiol 14(Suppl 1):92–97
116. Zvyagin AV, Zhao X, Gierden A, Sanchez W, Ross JA, Roberts MS (2008) Imaging of zinc oxide nanoparticle penetration in human skin *in vitro* and *in vivo*. J Biomed Opt 13:064031
117. Tsuji JS, Maynard AD, Howard PC, James JT, Lam CW, Warheit DB, Santamaria A (2006) Research strategies for safety evaluation of nanomaterials, part IV: risk assessment of nanoparticles. Toxicol Sci 89:42–50
118. NANODERM (2007) Quality of Skin as a Barrier to ultra-fine Particles. Final Report. Online available at http://www.uni-leipzig.de/~nanoderm/Downloads/Nanoderm_Final_Report.pdf
119. Larese FF, D'Agostin F, Crosera M, Adami G, Renzi N, Bovenzi M, Maina G (2009) Human skin penetration of silver nanoparticles through intact and damaged skin. Toxicology 255:33–37
120. Tinkle SS, Antonini JM, Rich BA, Roberts JR, Salmen R, DePree K, Adkins EJ (2003) Skin as route of exposure and sensitization in chronic beryllium disease. Environ Health Perspect 111:1202–1208
121. Wu J, Liu W, Xue C, Zhou S, Lan F, Bi L, Xu H, Yang X, Zeng FD (2009) Toxicity and penetration of TiO_2 nanoparticles in hairless mice and porcine skin after subchronic dermal exposure. Toxicol Lett 191:1–8
122. Powell JJ, Faria N, Thomas-McKay E, Pele LC (2010) Origin and fate of dietary nanoparticles and microparticles in the gastrointestinal tract. J Autoimmun 34:J226–J233
123. Hillyer JF, Albrecht RM (2001) Gastrointestinal persorption and tissue distribution of differently sized colloidal gold nanoparticles. J Pharm Sci 90:1927–1936
124. Kim YS, Song MY, Park JD, Song KS, Ryu HR, Chung YH, Chang HK, Lee JH, Oh KH, Kelman BJ, Hwang IK, Yu IJ (2010) Subchronic oral toxicity of silver nanoparticles. Part Fibre Toxicol 7:20–30
125. Semete B, Booysen L, Lemmer Y, Kalombo L, Katata L, Verschoor J, Swai HS (2010) *In vivo* evaluation of the biodistribution and safety of PLGA nanoparticles as drug delivery systems. Nanomedicine 6:662–671
126. IARC (1998) Asbestos. Monographs on the evaluation of carcinogenic risks to humans. overall evaluations of carcinogenicity: an updating of IARC monographs, vols 1–42, Suppl 7, World Health Organisation (WHO), International Agency for Research on Cancer (IARC), Lyon, France. Online available at http://monographs.iarc.fr/ENG/Monographs/suppl7/suppl7.pdf
127. IARC (1997) Silica. Summary of data reported and evaluation. Monographs on the evaluation of carcinogenic risks to humans, vol 68, World Health Organisation (WHO), International Agency for Research on Cancer (IARC), Lyon, France. Online available at http://monographs.iarc.fr/ENG/Monographs/vol68/volume68.pdf
128. Seaton A, MacNee W, Donaldson K, Godden D (1995) Particulate air pollution and acute health effects. Lancet 345:176–178
129. Oberdörster G (1988) Lung clearance of inhaled insoluble and soluble particles. J Aerosol Med 1:289–330
130. ICRP (1994) Human respiratory tract model for radiological protection. International Commission on Radiological Protection, ICRP Publication 66. Ann ICRP 24. Online available at http://www.icrp.org/publications.asp
131. Oberdörster G, Ferin J, Finkelstein J, Wade P, Corson N (1990) Increased pulmonary toxicity of ultrafine particles? II. Lung lavage studies. J Aerosol Sci 21:384–387
132. Oberdörster G, Ferin J, Lehnert BE (1994) Correlation between particle size, *in vivo* particle persistence, and lung injury. Environ Health Perspect 102(Suppl 5):173–179

133. Li XY, Brown D, Smith S, MacNee W, Donaldson K (1999) Short-term inflammatory responses following intratracheal instillation of fine and ultrafine carbon black in rats. Inhal Toxicol 11:709–731
134. Stoeger T, Reinhard C, Takenaka S, Schroeppel A, Karg E, Ritter B, Heyder J, Schulz H (2006) Instillation of six different ultrafine carbon black particles indicates a surface area threshold dose for acute lung inflammation in mice. Environ Health Perspect 114:328–333
135. Brown DM, Wilson MR, McNee W, Stone V, Donaldson K (2001) Size-dependent proinflammatory effects of ultrafine polystyrene particles: a role for surface area and oxidative stress in the enhanced activity of ultrafines. Toxicol Appl Pharmacol 175:191–199
136. Oberdörster G, Ferin J, Gelein R, Soderholm SC, Finkelstein J (1992) Role of the alveolar macrophage in lung injury: studies with ultrafine particles. Environ Health Perspect 97: 193–199
137. Oberdörster G, Sharp Z, Atudorei V, Elder A, Gelein R, Kreyling W, Cox C (2004) Translocation of inhaled ultrafine particles to the brain. Inhal Toxicol 16:437–445
138. Elder A, Gelein R, Silva V, Feikert T, Opanashuk L, Carter J, Potter R, Maynard A, Ito Y, Finkelstein J, Oberdörster G (2006) Translocation of inhaled ultrafine manganese oxide particles to the central nervous system. Environ Health Perspect 114:1172–1178
139. Ji JH, Jung JH, Kim SS, Yoon JU, Park JD, Choi BS, Chung YH, Kwon IH, Jeong J, Han BS, Shin JH, Sung JH, Song KS, Yu IJ (2007) Twenty-eight-day inhalation toxicity study of silver nanoparticles in Sprague-Dawley rats. Inhal Toxicol 19:857–871
140. Iavicoli I, Leso V, Fontana L, Bergamaschi A (2011) Toxicological effects of titanium dioxide nanoparticles: a review of *in vitro* mammalian studies. Eur Rev Med Pharmacol Sci 15:481–508
141. Khlebtsov N, Dykman L (2011) Biodistribution and toxicity of engineered gold nanoparticles: a review of *in vitro* and *in vivo* studies. Chem Soc Rev 40:1647–1671
142. Johnston HJ, Hutchison G, Christensen FM, Peters S, Hankin S, Stone V (2010) A review of the *in vivo* and *in vitro* toxicity of silver and gold nanoparticles: particle attributes and biological mechanisms responsible for the observed toxicity. Crit Rev Toxicol 40:328–346
143. Park EJ, Yi J, Chung KH, Ryu DY, Choi J, Park K (2008) Oxidative stress and apoptosis induced by titanium dioxide nanoparticles in cultured BEAS-2B cells. Toxicol Lett 180:222–229
144. Brown DM, Donaldson K, Borm PJ, Schins RP, Dehnhardt M, Gilmour P, Jimenez LA, Stone V (2004) Calcium and ROS-mediated activation of transcription factors and TNF-α cytokine gene expression in macrophages exposed to ultrafine particles. Am J Physiol Lung Cell Mol Physiol 286:L344–L353
145. Sayes CM, Reed KL, Warheit DB (2007) Assessing toxicity of fine and nanoparticles: comparing *in vitro* measurements to *in vivo* pulmonary toxicity profiles. Toxicol Sci 97: 163–180
146. Duffin R, Tran L, Brown D, Stone V, Donaldson K (2007) Proinflammogenic effects of low-toxicity and metal nanoparticles *in vivo* and *in vitro*: highlighting the role of particle surface area and surface reactivity. Inhal Toxicol 19:849–856
147. Handy RD, von der Kammer F, Lead JR, Hassellöv M, Owen R, Crane M (2008) The ecotoxicology and chemistry of manufactured nanoparticles. Ecotoxicology 17:287–314
148. Handy RD, Owen R, Valsami-Jones E (2008) The ecotoxicology of nanoparticles and nanomaterials: current status, knowledge gaps, challenges, and future needs. Ecotoxicology 17:315–325
149. Ono-Ogasawara M, Serita F, Takaya M (2009) Distinguishing nanomaterial particles from background airborne particulate matter for quantitative exposure assessment. J Nanopart Res 11:1651–1659
150. Rocks SA, Pollard SJ, Dorey RA, Harrison PTC, Levy LS, Handy RD, Garrod JF, Owen R (2009) Risk assessment of manufactured nanomaterials. In: Lead JR, Smith E (eds) Environmental and human health impacts of nanotechnology. Wiley, Chichester, pp 389–421
151. Morris J, Willis J, De Martinis D, Hansen B, Laursen H, Sintes JR, Kearns P, Gonzalez M (2011) Science policy considerations for responsible nanotechnology decisions. Nat Nanotechol 6:73–77

Immunotoxicology and Its Application in Risk Assessment

Andrew A. Rooney, Robert W. Luebke, MaryJane K. Selgrade, and Dori R. Germolec

Abstract Immunotoxicology is the study of undesired modulation of the immune system by extrinsic factors. Toxicological assessments have demonstrated that the immune system is a target following exposure to a diverse group of xenobiotics including ultraviolet radiation, chemical pollutants, therapeutics, and recreational drugs. There is a well-established cause and effect relationship between suppression of the immune response and reduced resistance to infections and certain types of neoplasia. In humans, mild-to-moderate suppression of the immune response is linked to reduced resistance to common community-acquired infections, whereas opportunistic infections, which are very rare in the general population, are common in individuals with severe suppression. Xenobiotic exposure may also result in unintended stimulation of immune function. Although a cause and effect relationship between unintended stimulation of the immune response and adverse consequences has yet to be established, evidence does suggest that hypersensitivity, autoimmunity, and pathological inflammation may be exacerbated in susceptible populations exposed to certain xenobiotics. Xenobiotics can act as allergens and

A. A. Rooney (✉)
National Toxicology Program, Office of Health Assessment and Translation, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA
e-mail: Andrew.Rooney@nih.gov

R. W. Luebke
National Health and Environmental Effects Research Laboratory, Cardiopulmonary and Immunotoxicology Branch, U.S. Environmental Protection Agency, Research Triangle Park NC, USA

M. K. Selgrade
Environmental Risk and Toxicology, ICF International, Durham, NC 27713, USA

D. R. Germolec
National Toxicology Program, Toxicology Branch, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA

A. Luch (ed.), *Molecular, Clinical and Environmental Toxicology*,
Experientia Supplementum 101, DOI 10.1007/978-3-7643-8340-4_9,

elicit hypersensitivity responses, or they can modulate hypersensitivity responses to other allergens such as pollen or dust mite by acting as adjuvants, enhancing the development or expression of hypersensitivity. Allergic contact dermatitis, allergic rhinitis, and asthma are the most commonly encountered types of hypersensitivity reactions resulting from chemical exposure. The immunologic effectors and mechanisms involved in autoimmune reactions are the same as those associated with responses to foreign antigens; however, the reactions are directed against the host's own cells. Thus, chemicals that induce immune suppression, nonspecific immunostimulation, or hypersensitivity may also impact autoimmunity. Risk assessment for immunotoxicity should be performed using the same approaches and principles for other noncancer effects. However, since xenobiotics may have effects on more than one aspect of immune function, immunotoxicity data should be evaluated separately for evidence of suppression, stimulation, hypersensitivity, and autoimmunity.

Keywords Allergic contact dermatitis · Autoimmunity · Benchmark dose · Buehler test · Guinea pig maximization test · Hypersensitivity · Human repeated insult patch test · Immunomodulation · Immunosuppression · Immunostimulation · Local lymph node assay · Respiratory sensitization · Skin sensitization · TCDD · Risk assessment · Weight of evidence

Introduction

Immunotoxicology is the study of undesired modulation of the immune system by extrinsic factors, thus potentially increasing the risk of infectious or neoplastic diseases, allergy/asthma, or autoimmune diseases. Toxicological assessments have demonstrated that the immune system is a target organ following exposure to environmental agents such as ultraviolet radiation, chemical pollutants, therapeutics, and recreational drugs (Table 1). Immunotoxicity testing guidelines have been established to assess potential immunosuppression [1–3], allergic skin sensitization [4–6], and autoimmunity [7], although the most abundant and reliable data available to risk assessors pertain to suppression and hypersensitivity. After providing a brief overview of the immune system, this chapter is organized around sections that review the basic concepts of immunotoxicology for the four major types of chemical-related immune effects (suppression, enhancement, hypersensitivity, and autoimmunity) and their application to risk assessment.

The Immune System and Immune Function

The primary function of the immune system is to destroy or neutralize infectious agents and certain tumor cells. Immune cells are located throughout the body, either in distinct organs, including the spleen, thymus, and lymph nodes, or in diffuse

Table 1 Agents associated with immunotoxicity

Agents	Immunomodulation[a]	Hypersensitivity[b]	Autoimmunity[a]
Metals			
Lead	S, E	W	E
Mercury	S, E		E
Platinum (halogenated salts)		D	
Nickel	S	D	
Oxidant gases			
Nitrogen dioxide	S, E	W	
Ozone	S, E	W	
Persistent organic pollutants[c]			
Polychlorinated biphenyls	S, E		E
Hexachlorobenzene	S, E		E
Pesticides			
Chlordane	S, E		
Malathion	S, E		E
Tributyltin oxide	S		
Pharmaceuticals			
Cyclosporine A	S		
Halothane	E		E
Isoniazid			E
Cyclophosphamide	S, E		E
Polyisocyanates			
Toluene diisocyanate (TDI)		D	
Recreational drugs			
Cocaine	S		
Ethanol	S		
Tobacco smoke	S, E	D, W	E
Solvents			
Benzene	S		
Trichloroethylene	S, E	D	E
Vinyl chloride	E		E

[a]Suppression (S) or enhancement (E) of immune function or of symptoms of autoimmunity
[b]Direct (D) sensitization/elicitation or worsening (W) of hypersensitivity
[c]Persistent organic pollutants not commonly used as pesticides

accumulations of lymphoid and myeloid cells associated with the mucosal surfaces of the urogenital and GI tracts, skin, and lung. These tissues are the primary sites for the immune system to detect entering pathogens and exogenous proteins. Immune responses driven by proteins or carbohydrates (antigens) unique to a particular pathogen or cell are referred to as antigen-specific or adaptive, whereas responses to genetic material or structural components that are similar in a wide range of organisms (e.g., viral double-stranded RNA or components of bacterial cell walls) are referred to as nonspecific or innate.

Innate immune responses are able to provide a rapid defense against pathogenic organisms because cells and molecules that mediate innate immunity do not require antigen recognition or cell division/maturation. Effector cells include macrophages (phagocytosis and killing of pathogens, antigen processing, production of proinflammatory mediators), dendritic cells (antigen processing and presentation to lymphocytes, cytokine production), natural killer (NK) cells (killing of certain

tumor cells and pathogens), and polymorphonuclear (PMN) leukocytes (phagocytosis and killing of bacteria). Soluble mediators participate in innate responses as well; components of the complement cascade lyse cells and augment phagocytosis of bacteria and various cytokines upregulate inflammation. However, cells and soluble mediators of the innate response also contribute to the development of adaptive immune responses via activation and regulation of other lymphocyte subpopulations.

The adaptive immune response entails recognition of foreign antigens presented to cell surface receptors on lymphocytes by innate antigen processors, followed by gene transcription, production of growth factors, clonal expansion of antigen-specific lymphocytes, activation of effector mechanisms that are ultimately directed against infectious agents or neoplastic cells, and the generation of long-lived memory cells. The response is target specific but develops slowly, peaking after 5–10 days. Lymphocytes are the principal cellular effectors of adaptive responses, classified first by their tissue of origin, and then by function or maturational state. Bone marrow-derived lymphocyte progenitors which mature in specific regions of the bone marrow, spleen, and lymph nodes are referred to as B lymphocytes or B cells. These B cells secrete antibodies, which are the soluble mediators responsible for humoral immunity. Cell-mediated immunity, on the other hand, is coordinated by the other major class of lymphocytes, T cells or T lymphocytes. T cells are derived from the common lymphocyte progenitor population that migrate to and mature in the thymus. T lymphocyte subpopulations include cells that assist in and amplify other immune responses (T helper cells; T_H), downregulate other immune responses (T suppressor cells; T_S), or destroy infected or neoplastic cells (cytotoxic T cells; T_C). T_H cells produce cytokines that regulate immune function and can be further subdivided into subpopulations which assist other T cells (T_H1) or which stimulate and perpetuate antibody responses (T_H2). T_H2 cytokine production predominates in newborns, and the release of these cytokines is associated with increased allergy and asthma.

Antigen-naïve circulating B cells encounter antigen in lymph nodes or tissue-associated lymphoid tissues via membrane-bound immunoglobulin (Ig) molecules that act as antigen receptors. Receptor cross-linking initiates a signal transduction cascade that, with the appropriate stimulus from T_H2 cytokines, leads to activation, clonal expansion and differentiation into antibody-secreting plasma cells, and a small population of long-lived memory cells. The five classes of antibodies, IgM, IgG, IgE, IgA, and IgD, are distinguished by the characteristics of their heavy chain polypeptides, and each class has distinct expression patterns and functional properties. IgM and IgG antibodies are frequently measured in studies of immune function for potential suppression, while increased total and antigen-specific IgE may indicate allergic hypersensitivity responses. Although IgA has a central role in mucosal immunity, neither IgA nor IgD is routinely evaluated.

Unintended Suppression or Enhancement of Immune Function

Suppression and stimulation of immune function are conceptually and biologically distinct, although when either occurs following xenobiotic exposure, the outcome is generally considered adverse. Unintended immunosuppression is universally recognized as adverse because immunosuppressed individuals are more susceptible to infections and certain types of malignancies. As a result, testing and regulatory strategies were designed to detect and characterize suppression of the immune response. In contrast, stimulated immune function is usually perceived as beneficial, as exemplified by the use of adjuvants to improve responses to vaccination. However, enhancement and suppression are not mutually exclusive events, particularly when the underlying mode of immunotoxicity is modulation of regulatory cytokines that drive either inflammatory or antibody responses to antigens. In this situation, the apparent outcome of hazard identification testing (change *vs*. no change) will depend on which immune functions are evaluated. Thus, failure to consider unintended stimulation of immune function as potentially adverse may result in disregarding data that indicate exposure-related effects with the potential to impact inflammation and disease.

Suppression

Studies dating from the 1970s conclusively established that exposure to diverse chemical classes adversely affects immunocompetence (Table 1) [1–3, 8, 9]. In humans, mild-to-moderate suppression reduces efficacy of vaccines and increases the likelihood of common community-acquired infections, but does not affect the incidence of opportunistic infections that typically accompany severe forms of immunodeficiency diseases [10, 11]. Maternal exposure to compounds such as polychlorinated biphenyls (PCBs) during the period of fetal immune system development may result in persistent immunosuppression in the offspring, at doses that do not affect adult immune function [1, 12].

Hazard Identification and Assessment of Immunosuppression

Clinical and Epidemiological Data

Functional assessment of the human immune system is technically simple and may be accomplished by monitoring the primary antibody response to vaccination or delayed-type hypersensitivity response to naturally occurring antigens in adults or infants [13, 14]. A description of biomarkers useful in the evaluation of immunity in epidemiological studies is provided in Report of the Bilthoven Symposium: Advancement of Epidemiological Studies in Assessing the Human Health Effects

of Immunotoxic Agents in the Environment and the Workplace [15]. Although biomarkers are available, it is important to note that detection of mild-to-moderate immunodeficiency may be difficult in humans using common assays (e.g., leukocyte counts, immunoglobulin levels) because the range of "normal" is large. Likewise, identifying a small exposure-related change in infectious disease incidence is also difficult [16, 17]. Researchers have linked low-to-moderate degrees of immunosuppression and increased infectious disease incidences with chronic psychological factors including separation and divorce, caregiving for Alzheimer's patients, and bereavement [11].

Chronic stress has been linked to reactivation of latent viruses, such as CMV, HSV-1, or EBV, as measured either by reoccurrence of symptoms or by elevations in specific antibody titer [18–24]. Elevated antiviral antibody titer signals viral reactivation and replication and precedes disease onset, although only about 20% of those with elevated titers actually develop clinical disease. The immunosuppressive effects of stress have been confirmed in a controlled infectious challenge study in humans [25].

Animal Data

Animal immunotoxicity data are generated as the result of regulatory mandate or by research conducted by government, academic, and commercial laboratories and may include relatively insensitive markers (lymphoid organ weights, blood cell counts), functional assessment (e.g., the response to immunization), or, rarely, assessment of resistance to infection or tumor challenge (i.e., host resistance assays). Host resistance is typically the standard against which other assays are judged because altered resistance is a biologically plausible effect with clear relevance for potential adverse effects in humans. However, challenge agents in host resistance assays should be chosen to explore or confirm a known functional defect, not used to screen for effects. Nonlethal resistance models, in which numbers of tumor foci, viral titers, or bacterial counts in target tissues are assessed, provide greater sensitivity than mortality because the data provide a quantitative assessment of the host response to challenge and are a better reflection of protective immunity in the organism. Furthermore, the biological relevance of death as an endpoint is questionable when most or all of the immunocompetent controls do not survive because the virulence or number of the challenge agents simply overwhelms the initial response to infection, killing the host before a protective response can be mounted.

If host resistance data are not available, potential effects on host resistance must be inferred from other immune endpoints. The predictive power of commonly used assays for altered host resistance assays varies although concordance rates may reach 100% when data from multiple assays are combined [16, 17]. Common endpoints and methods used to assess immunosuppression are described in the International Programme on Chemical Safety (IPCS) Environmental Health Criteria 180: Principles and Methods for assessing Direct Immunotoxicity with

Exposure to Chemicals [26]. The document also provides context and information that may assist in the interpretation of immunosuppression data for risk assessment. The Risk Assessment section of this chapter includes additional discussion of important issues to be considered when evaluating immunotoxicity data.

Stimulation

Deliberate stimulation of the immune system is a routine and mostly beneficial clinical procedure. Chemical and/or biological adjuvants are routinely included in vaccines to increase and prolong the immune response and to improve the response to weak antigens; successful induction of protective immunity is often dependent on their activity. However, failure to control the intensity and duration of normally protective immune responses, as a consequence of inherited defects, an ongoing disease process, or chemical exposure, is a well-documented cause of immune-mediated tissue damage.

Adverse effects associated with stimulation of the immune system by a xenobiotic include inappropriate stimulation or skewing of normally protective immune responses, direct allergenicity of the xenobiotic, induction or worsening of autoimmune disease, and nonspecific inflammation. Inappropriate stimulation of responses to infectious agents that are normally protective may increase inflammation, resulting in excess tissue damage or the potential of exposing cryptic host antigens (self-antigens the host normally does not encounter), one possible pathway to autoimmune disease. Skewing of the immune response to favor either inflammation or allergy can also occur; some studies have linked such changes with an increased risk of developing allergies and reduced resistance to certain infectious agents. Hypersensitivity and autoimmune disease are covered elsewhere in this review. Inflammation is a normal component of tissue injury associated with toxicant exposure. This type of injury is generally considered in the context of specific organ systems and will not be discussed as part of this chapter. This section will examine the evidence that unintended stimulation of either the innate or adaptive immune responses should be considered an adverse effect and taken into account in a risk assessment.

Hazard Identification and Assessment of Immunostimulation

Regulatory acknowledgement of unintended immune system stimulation as an adverse effect is limited. However, the US Food and Drug Administration (FDA) Immunotoxicity Testing Guidance [27] and the FDA Guidance for Industry on Immunotoxicology Evaluation of Investigational New Drugs [28] both list unintentional immunostimulation as a category of immunotoxicity with potential adverse effects. The FDA [27] considers adverse immunostimulation to include unintentional immunogenicity and adjuvant activity and warns that “nonspecific enhancement

of the immune response that might be interpreted as a beneficial effect may result in suppression of specific immunity against a particular infection." The FDA also states that unintended stimulation may result in autoimmunity, hypersensitivity, and chronic inflammation.

Clinical and Epidemiological Data

Clinical data suggest that moderate stimulation of the immune system by adjuvant is not associated with immune-mediated disease in the general population, although adverse effects have been reported in a few individuals with preexisting autoimmune disease. For example, the use of "immunostimulating" herbal supplements has been temporally associated with flare-up of pemphigus vulgaris in two patients and with the onset and later flare-up of dermatomyositis in another [29]. Nevertheless, human studies suggest that routine vaccination against influenza and pneumonia is safe and effective in patients with various systemic autoimmune diseases, in spite of previously expressed concerns that immune system activation by components of the vaccine may activate or worsen systemic autoimmune disease [30–32].

In contrast, there are also several examples in which unintentional stimulation of human immune function by chemical exposure is known to cause adverse effects. Occupational exposure to silica stimulates innate immune system cells and is associated with development of human autoimmune disease [33]. Likewise, unintended upregulation of human antibody production by mercury is suggested by a study in which removal of dental amalgam from individuals with autoimmune thyroiditis and mercury hypersensitivity was determined to reduce autoantibodies to thyroglobulin and thyroid peroxidase [34].

Disruption of immune system balance by suppression of certain immune functions could theoretically lead to enhancement of others. *In utero* exposure to cigarette smoke has been strongly associated with increased risk of developing asthma [35, 36], yet animal data demonstrated that such exposures also increased susceptibility to tumor challenge via persistent suppression of cytotoxic T cell activity [37]. Exposure to PCBs was associated with less shortness of breath and wheeze [38], whereas immune suppression and increased infection were observed in similarly exposed populations [39, 40]. Given the immunomodulatory properties of some hormones, chemical disruption of endocrine system may also have adverse effects on immune function and balance in the immune system.

In aggregate, the literature suggests that inadvertent stimulation of the immune response may have adverse effects. In many cases, genotype appears to be a significant risk factor for the development of adverse effects, suggesting that sensitive individuals, rather than the general population, are at greater risk.

Animal Data

Immunotoxicity studies have consistently determined that exposure to various classes of xenobiotics and certain drugs is associated with increased or enhanced immune function, particularly the T cell-dependent antibody response (TDAR). In many cases, these same compounds were determined to hasten disease onset or enhance disease severity in genetically susceptible models. However, fewer reports describing the risk posed by unintended immunostimulation in nonsusceptible animals are available.

Unlike immune suppression, well-characterized methods to assess immunostimulation in rodent models are not widely available. However, a number of studies have shown that respiratory exposure to air pollutants (NO_2, ozone, residual oil fly ash, and diesel exhaust) enhances both allergic sensitization to common allergens such as dust mite antigens and enhances respiratory responses to allergen challenge [41–43]. In addition, oxidant gases (e.g., ozone) have been reported to enhance immune pathology associated with influenza infection [44]. Other routes of exposure may produce similar effects; dioxin [45, 46] and ultraviolet radiation [47] decrease host resistance to influenza infection apparently by enhancing inflammation rather than suppressing immune defenses.

As noted above, the TDAR is widely used to detect suppression of immune function, and unexpected enhancement of the antibody response is also associated with adverse effects, particularly in susceptible subpopulations. For example, lead (Pb) exposure exacerbates autoimmune disease in a genetically prone mouse model of lupus, but does not induce disease in resistant strains of mice [48]. Rodgers *et al.* [49] reported that a single high but noncholinergic dose of the insecticide malathion increased the number of spleen cells of C57Bl/6 mice producing IgM antibody to sheep red blood cells, in the absence of changes in spleen weight or cellularity or cholinergic activation. A later study [50] determined that oral exposure to malathion (33–300 mg/kg/week, beginning at 6 weeks of age) accelerated the onset of autoimmune disease and increased autoantibody production in autoimmune-prone mice (MRL-lpr; a model of spontaneous systemic lupus erythematosus or SLE), but not in congenic-resistant mice (MRL +/+). Although high concordance between suppression of the IgM response and susceptibility to infectious disease has been demonstrated, it is unknown whether similar concordance exists between increased IgM responses and disease. The same is true for other functional endpoints (e.g., delayed-type hypersensitivity, NK cell activity).

Suppression and Enhancement Conclusions

There is a well-established cause and effect relationship between suppression of the immune response and reduced resistance to infections and certain types of neoplasia. In humans, mild-to-moderate suppression of the immune response is linked to reduced resistance to common community-acquired infections, whereas

opportunistic infections, which are very rare in the general population, are common in individuals with severe suppression. Quantitative functional data (e.g., responses to novel antigens) are generally predictive of resistance to infectious agents and tumor cells and provide insight into the potential consequences of suppression. Host resistance assays confirm that exposure increases the risk of infection, although models that rely on mortality as an endpoint may be misleading, if the dose or virulence of the challenge agent is not chosen with care. In contrast, a cause and effect relationship between unintended stimulation of the immune response, observed when testing for reduced function, and adverse consequences has yet to be established. Nevertheless, associative data suggest that hypersensitivity, autoimmunity, and pathological inflammation may be exacerbated in susceptible populations. As such, elevated responses in experimental groups of animals, immunized during tiered testing for immunotoxicity, should not be ignored, as it is a clear indication that modulation of the immune system has occurred. The final decision on how to interpret the data is a policy decision; the regulatory mandate, and the ability of the risk assessor to ask for additional testing, will determine how, or if, additional testing will be pursued when unintended stimulation is detected.

Hypersensitivity

Hypersensitivity (allergy) is defined as excessive humoral or cellular immune responses to an otherwise innocuous antigen, which can lead to tissue damage. Xenobiotics can pose a risk of allergic disease in one of two ways: they can act as allergens and elicit hypersensitivity responses, or they can modulate hypersensitivity responses to other allergens such as pollen or dust mite antigens by acting as adjuvants, enhancing the development or expression of hypersensitivity responses. This discussion will focus on chemical allergens. Chemicals that act as allergens include certain proteins that can by themselves induce an immune response and low molecular weight chemicals (known as haptens). Haptens are physically too small to induce a specific immune response but are chemically reactive and covalently bind to larger molecules, usually a protein, to form chemical-protein moieties of sufficient size to induce an immune response that is then hapten specific. For more details on the mechanisms underlying hypersensitivity reactions in general and allergic contact dermatitis (ACD) and respiratory allergies in particular, see Selgrade *et al.* [51].

ACD and hypersensitivity responses in the respiratory tract, including allergic rhinitis and asthma, are the most commonly encountered types of allergy resulting from occupational, consumer, and environmental exposures to chemicals [52]. In addition, certain drugs as well as certain food components are associated with systemic anaphylactic reactions [53]. Methods for hazard identification are very limited in the latter case; hence, this review will focus on ACD, for which there are widely accepted test guidelines [54], and hypersensitivity responses in the respiratory tract. Evidence for risk to the respiratory tract is largely based on epidemiology although experimental animal models have been used in research.

Hypersensitivity reactions to chemicals pose some particularly challenging problems for quantitative risk assessment because they develop in two separate stages: (1) induction and (2) elicitation. Induction (sensitization) is the first step and requires a sufficient single or cumulative exposure dose of the sensitizing agent to induce immune responses (i.e., development of antigen-specific antibodies or T cells). Obvious symptoms are generally not observed at this stage. Elicitation occurs in sensitized individuals upon subsequent exposure to the antigen and results in adverse responses that include inflammation. The dose responses for these two stages are different although not entirely independent [55–57]. Usually, the dose required for sensitization is higher than that required for elicitation. However, when the induction dose is high, the dose required to elicit a response is lower than that required when the induction dose is low. In practice, it is sometimes difficult to determine the point at which sensitization ends and elicitation begins. For this reason, risk assessments for hypersensitivity have been largely limited to hazard identification in which some chemicals are labeled as sensitizers, but without any indication of potency. However, significant progress has been made recently, particularly with ACD, in the development of dose–response relationships and thresholds. Various *in vivo* approaches to qualitative and quantitative risk assessment will be reviewed in this chapter. Because of animal welfare concerns and requirements imposed by the chemical legislation in Europe (e.g., 7th Amendment of the Cosmetics Directive and REACH), there is an increasing emphasis on the development of *in vitro* methods for hazard identification and potency characterization. This topic will be covered in another chapter of this book (see chapter *Chemical Sensitization and Allergotoxicology*, authored by Van Den Heuvel *et al*.).

Hazard Identification and Assessment of Skin Sensitization

The most familiar skin-sensitizing agent is poison ivy. However, ACD is one of the most commonly occurring occupational skin diseases [58], and fragrances, cosmetics, personal care products, reactive dyes, metals, preservatives, and pesticides all have the potential to cause skin sensitization. To insure consumer product and workplace safety, test guidelines have been developed and refined for the identification of chemicals that have the potential to induce hypersensitivity responses in the skin.

Induction

Clinical and Epidemiological Data

Experimental sensitization studies with human subjects have been used to provide dose–response information on the induction of ACD. These studies involve repeated application of the chemical to the skin under a patch, followed by a rest

period and then a challenge exposure. Methods vary with respect to subject number, skin site, type and number of induction patches, patch application time, time between induction and challenge, and challenge duration. In all cases, enhancement of the skin response after challenge over that seen during early induction exposures has been the criterion by which induction of contact allergy is measured. The test most typically conducted is the human repeated insult patch test (HRIPT) [59]. A similar test, the human maximization test (HMT) found in earlier papers, differs from the HRIPT in that the patch application is to irritated skin. For this reason, HMT is not in common practice today although results from earlier studies may be available to risk assessors [60]. The readout for use in dermal sensitization risk assessments is usually incidence of sensitization. When HRIPTs are done using several different induction doses, a dose–response curve (induction incidence *vs.* dose expressed as amount of chemical applied per area of skin) and effect levels can be derived (i.e., the no-observed-effect-level or NOEL and the lowest-observed-effect-level or LOEL).

Today, for ethical reasons (i.e., the risk of sensitizing a previously naïve individual), skin sensitization hazard is usually assessed using an animal model (see below) such as the mouse local lymph node assay (LLNA), guinea pig maximization test, or guinea pig Buehler test. The HRIPT is sometimes employed as a confirmatory assay to substantiate the lack of sensitization at an exposure level which was identified as a NOEL in an animal model or which was derived as a likely NOEL from quantitative structure–activity relationships [61]. Epidemiological data documenting sensitization, usually from occupational cohorts, may also be available. The prevalence of acute contact dermatitis in a population exposed to a particular substance may be used in hazard identification and may also provide dose–response information, NOEL, LOEL, and benchmark dose (BMD), if adequate exposure data, expressed as skin area dose, are available.

Animal Data

Several testing strategies using laboratory animals are available to evaluate the potential for chemicals to induce contact sensitization. Historically, guinea pig models have been used. These methods rely on the induction of sensitization in animals and then the subjective evaluation of erythema and edema following test article challenge at a naïve site. Two of the most commonly used methods are the Buehler test [62] and the guinea pig maximization test [63]. In recent years, the mouse LLNA [64] has undergone extensive development and validation and was peer reviewed by the Interagency Coordinating Committee on the Validation of Alternative Methods [65, 66]. This assay evaluates only the induction phase of contact hypersensitivity and uses quantitation of ^{3}H-thymidine incorporation into proliferating draining lymph node cells (clonal expansion) to evaluate the sensitization potential of chemicals. The LLNA has a number of advantages over the guinea pig assay including the use of a quantitative endpoint based on the

relevant mode of action, acquisition of dose–response data, reduction in the number of animals used, lack of interference when testing colored materials, and a reduction in animal distress and the time required to conduct a study. By evaluating the dose response, this assay provides data that can be used to calculate an EC3 (the chemical exposure concentration required to induce a threefold increase in stimulation over control), which can then be used to rank the potency of the chemical.

A number of studies that compared human NOELs and BMDs with LLNA thresholds (EC3 values) indicate that area doses are directly comparable between mice and humans [67–69]. Therefore, the LLNA EC3 value has been suggested as a surrogate NOEL in quantitative risk assessment. In contrast, the traditional guinea pig tests are not as amenable to relative potency/quantitative assessments. Recently, guinea pig protocols have been modified in order to generate such data [70–72], but these protocols have not yet been validated. Hence, for *de novo* investigations, the LLNA is the animal test method best suited for quantitative estimations of skin-sensitizing potency [73].

Elicitation

Clinical and Epidemiological Data

The elicitation dose–response relationship for ACD can be determined in human studies using subjects that already have an established allergy. However, these data are likely to be rare because diagnostic tests frequently employ a single, relatively high concentration in a patch test [74] because the goal is to identify the sensitizer rather than determine the NOEL/LOEL concentration for elicitation. The concentration of the sensitizer (in a suitable vehicle such as petroleum jelly) can easily be varied in a patch test to determine a minimum elicitation threshold (MET), e.g., inducing a response in 10% of the subjects tested (MET10). Alternatively, the repeated open application test (ROAT) [75] can be employed in which formulations with different concentrations of the sensitizer, as well as a control formulation without the sensitizer, are employed and a NOEL or BMD determined. There is good correlation between the results from the patch test and the ROAT [76]. As noted earlier, the induction and elicitation dose responses are not entirely independent, and there is not a clear demarcation between induction and elicitation. Exposure can continue to contribute to induction even as elicited responses are occurring, and therefore, subsequent elicitations could occur at lower doses. Hence, it is best to determine elicitation thresholds in well-established rather than newly allergic subjects because it is assumed that induction reaches a maximum (levels off) at some point.

Animal Data

As discussed above for clinical endpoints, elicitation thresholds in newly sensitized animals depend on the frequency and dose used for sensitization. Elicitation NOELs are usually not determined in animal studies, because long-term exposure protocols that might address this issue have not been developed.

Hazard Identification and Assessment of Respiratory Sensitization

Although various types of immune-mediated injury can occur in the lung as a result of chemical exposure [52], those that cause rhinitis and asthma via IgE- (sometimes referred to as atopy) and T_H2-cell-mediated responses are of particular concern. The incidence of atopic disease and asthma in the USA increased dramatically between 1980 and 1995 and remains a concern today [77], particularly in children. A similar trend also appeared in other westernized nations [78]. Also, work-related asthma is the most commonly reported occupational lung condition [79]. Allergic asthma can result from exposure to proteins (frequently enzymes) or from exposure to low molecular weight chemicals that, as with contact sensitizers, must be chemically linked with a protein carrier in order to sensitize the immune system (see Table 2 for example compounds).

Currently, there are no universally accepted animal models for human risk of respiratory sensitization that permit the determination of the dose–response relationship or relative potency of enzymes or low molecular weight chemicals for allergen-specific antibodies or symptoms of allergy via the inhalation route. Given the importance of IgE for many cases of respiratory sensitization, the generation of total IgE or antigen-specific IgE or cytophilic (usually predominantly IgE) serum antibodies is often used as a biomarker of disease to obtain threshold or benchmark value in both animal and human studies. Although the presence of IgE antibody does not equate to disease, it does increase the risk for development of allergy symptoms, but there are no absolute relationships between the levels of IgE antibody and symptoms. In the case of detergent enzymes, more intense exposures have been associated with symptoms, while less intense exposures have been associated with production of allergen-specific antibodies [83]. Both the occupational and guinea pig data indicate that there are thresholds for the induction of antibodies and for the elicitation of symptoms [84].

Whereas there is no doubt that IgE antibody plays an important role in respiratory allergy to proteins, there is some debate about the relevance of IgE antibody for the development of occupational asthma in response to chemical allergens. Although chemical respiratory allergens induce specific IgE in some symptomatic subjects, about half of subjects with allergy and asthma associated with diisocyanates do not exhibit this response and have late-onset (possibly IgG- or cell-mediated) responses in the absence of an immediate response [85–87]. Similarly, a small portion of subjects with asthma induced by acid anhydrides also exhibit

Table 2 Representative respiratory sensitizers for which dose–response data exist (according to [80–82])

Chemical	Induction	Elicitation
Proteins		
Enzymes		
Alcalase	X	X
Amylase	X	
Microbial extracts/components/products		
Dust mite	X	X
Cockroach	X	
Various mold species	X	
Plant materials		
Wheat	X	X
Latex	X	X
Red cedar		X
Animal		
Cat allergen	X	
Rat allergen/lab animals	X	X
Low molecular weight compounds		
Polyisocyanates		
Toluene diisocyanate (TDI)	X	X
Diphenylmethane-4,4′-diisocyanate (MDI)	X	X
Acid anhydrides		
Trimellitic anhydride (TMA)	X	X
Hexahydrophthalic anhydride (HHPA)	X	
Methylhexahydrophthalic anhydride (MHHPA)		X
Metals		
Halogenated platinum salts	X	

late-onset responses (associated with IgG) in the absence of immediate responses [88, 89]. Hence, some portion of disease could be missed using IgE exclusively as the biomarker.

Induction

Clinical and Epidemiological Data

Dose–response data on the induction of respiratory sensitization may be obtained from epidemiologic studies that are either designed as a prospective study (with new workers, or when a new compound is introduced in the workplace or consumer market) or as a retrospective study (often involving experimental measurement of reconstituted exposure scenarios). There are several examples, both for protein and low molecular weight chemicals, where no observed effect concentration (NOEC) or benchmark concentrations have been derived (based on respiratory symptoms and/or cytophilic antibody responses assessed by skin prick or serum testing) (see Table 2) [80, 81, 90–92]. However, sufficient data for determination of the dose response for sensitization are available only for a very limited number of allergens.

Animal Data

Guinea pig and mouse models have been used to study both protein and low molecular weight chemical sensitizers. For protein allergens, such as detergent enzymes, animal dander, and biotechnology products, induction of cytophilic antibodies in experimental animals (IgE in mouse and rat and IgG1 in guinea pigs) following intratracheal or involuntary aspiration exposure has been used successfully for both hazard identification and to develop dose–response data. The best example comes from the detergent industry [84]. In these studies, guinea pigs and more recently mice have been used to establish the relative potency for respiratory sensitization of different enzymes based on the dose response. The endpoint measured was enzyme-specific, serum, cytophilic antibodies (IgE and IgG1). Subtilisin was chosen as the reference allergen because the American Conference of Government and Industrial Hygienists developed a threshold limit value in the workplace for subtilisin A of 60 ng protein/m^3 based on historic human data, and the industry itself had an occupational exposure guideline of 15 ng protein/m^3 that prevented induction of occupational asthma in their workforce. By comparing new enzymes to this reference allergen, it was possible to use animal serum antigen-specific cytophilic antibody levels to set safe exposure concentrations for humans [93].

Dose responses and thresholds for both immune and respiratory endpoints characteristic of asthma were also demonstrated in mice exposed by involuntary aspiration to mold extracts. Differences in potency between microbial extracts were demonstrated [82, 94, 95]. However, because these exposures included all the mold proteins extracted, it is not possible to draw conclusions about administered dose of specific allergens. In the case of low molecular weight compounds, dose–response data and no effect levels have been demonstrated for toluene diisocyanate (TDI) exposures using a guinea pig model and cytophilic antibody as the endpoint. Exposure to higher TDI concentrations resulted in both a greater percentage of animals producing antibodies and higher antibody titers. Pulmonary sensitivity, assessed by bronchial provocation challenge with TDI conjugated to protein, also demonstrated no effect at the same exposure level [96]. Although NOELs or benchmark concentrations can be derived from inhalation studies in experimental animal such as those described above, there are significant uncertainties that result from the lack of standardized guidelines. The length of the daily exposure, number of exposure days, and the challenge concentration and endpoints assessed are all variables that can influence results. Also, both the detergent matrix and components of the microbial extracts may have adjuvant effects that can facilitate induction [84, 97]. Thus, experimental exposures are best conducted using a matrix that closely mimics environmental exposures.

As the studies cited here indicate, it is commonly assumed that allergic sensitization of the respiratory tract results from inhalation exposure to the allergen. Recently, animal studies have suggested that sensitization, at least to low molecular weight chemicals, can occur following dermal exposure and that both immunologic and respiratory responses characteristic of asthma have been observed [98–100].

The possibility that dermal exposure might be an alternative route of sensitization or that more than one route of exposure could contribute to the sensitization complicates efforts to develop quantitative assessments.

Elicitation

Clinical and Epidemiological Data

Human data derived from epidemiological studies or experimental/diagnostic provocation tests can be used to develop dose–response relationships and thresholds for the elicitation of respiratory allergy. However, in practice, data are available for only a handful of low molecular weight chemical and protein exposures (Table 2). Occupational studies have reported thresholds for organic acid anhydrides and for isocyanates [80, 101]. For high molecular weight antigens, thresholds have been reported for wheat flour, natural rubber latex, western red cedar, and rat allergens [102]. For both proteins and low molecular weight chemicals, the prevalence of sensitized workers experiencing symptoms, the frequency of symptoms, and their severity (e.g., measured as force expiratory volume in 1 s — FEV_1) were correlated with the mean exposure concentrations of antigen in air [80]. Although data are not available on the relationship between the induction and elicitation dose responses for respiratory sensitization, it is likely that as with skin sensitization the two are interdependent, and therefore, it would be best to determine elicitation thresholds in well-established rather than newly allergic subjects.

Animal Data

Because elicitation thresholds in newly sensitized animals likely depend on the frequency, dose, and route used for sensitization and because there are no standardized animal models for respiratory allergy, it is difficult to interpret elicitation dose responses in the few studies where they are described. Most data on elicitation after inhalation exposure obtained in animal models of respiratory sensitization were obtained after a single or a few induction exposures, rather than after long-established respiratory sensitization, and induction was often done by injection [103, 104] or dermal application [105–107] of the test substance. Also, in some cases, animals sensitized to low molecular weight chemicals were challenged with chemical-protein adducts, rather than free chemical.

Hypersensitivity Conclusions

In conclusion, data exist that can be used to assess the risk of inducing or triggering (eliciting) allergic responses following exposure to both protein and low molecular weight (hapten) chemicals. For ACD, standardized test methods and a large body of

data have been developed. For respiratory sensitization, standardized tests do not exist, and the database is much smaller. In both cases, there are considerable uncertainties surrounding the relationship between induction and elicitation doses, the timing of induction and elicitation exposures (both duration and the interval between), the contribution of the exposure matrix to the response, and the role that genetic susceptibility plays (particularly in the case of respiratory responses to proteins).

Autoimmunity

Autoimmunity and autoimmune diseases result from immune responses against self-molecules. The immunologic effectors and mechanisms involved in autoimmune reactions are the same as those associated with responses to foreign antigens, including activation of the innate and adaptive immune systems, production of inflammatory mediators, and activation of T lymphocytes or the generation of antibodies. However, in the case of autoimmunity, the response is directed to self-antigens. Thus, chemicals that induce immune suppression, nonspecific immunostimulation, or hypersensitivity may also impact autoimmunity. In many instances, the events that initiate the immune response to self are unknown although specific gene polymorphisms, gender-related hormones, and exposures to certain therapeutic drugs, bacteria, and viruses have been shown to be associated with the induction, development, or exacerbation of autoimmunity. In some cases, the causal link between bacterial or viral infection and autoimmunity has been fairly well established. Many peptide fragments of microbial agents are homologous with host proteins, and the induction of an immune response to these antigens can result in cross-reactivity with self-antigens and the induction of autoimmunity. A number of chemicals and therapeutic agents have been identified as potential triggers for autoimmunity and have been suggested to both induce onset and modulate disease severity [7, 108]. While it is believed that a genetic predisposition to self-reactivity exists in all individuals with autoimmune diseases, differing susceptibility factors may govern the timing or specific disease an individual develops. As with other multifactorial diseases, such as cancer, it is suggested that both genetic and environmental factors interact to determine disease outcome and progression; however, we have little knowledge with regard to whether they result in cumulative and sequential changes or are the sequelae of mixtures of exposures.

Autoimmune disorders can affect virtually any site in the body and present as a spectrum of diseases ranging from organ specific, in which antibodies and T cells react to self-antigens localized in a specific tissue, to systemic, characterized by reactivity against a specific antigen or antigens present in various tissues. Organ-specific autoimmune diseases are typically characterized by cell-mediated immune responses directly affected by autoreactive CD8+ (cytotoxic) T_C cells or indirectly via release of proinflammatory cytokines and other soluble mediators by activated CD4+ T_H cells and macrophages. In contrast, systemic autoimmune diseases are

generally characterized by specific autoantibodies, which can cause injury via activation of complement, blocking or stimulating cell surface receptors, or by aggregation into immune complexes that activate nonspecific inflammatory responses.

Hazard Identification and Assessment of Autoimmunity

Clinical and Epidemiological Data

Recent estimates suggest that 3–5% of the general population suffers from autoimmune diseases, and there is epidemiological evidence that the prevalence of certain autoimmune diseases is increasing in industrialized countries [7]. It has also been suggested that a number of common health problems, such as atherosclerosis, inflammatory bowel disease, and aspects of male and female infertility, may have an autoimmune component. The incidence of some autoimmune diseases has a clear gender bias, indicating a potential role for estrogens, androgens, and/or gonadotropins. Women have a significantly higher risk of developing an autoimmune disease than men, and in a majority of the most common autoimmune diseases (thyroiditis, scleroderma, systemic lupus erythematosus (SLE), multiple sclerosis (MS), rheumatoid arthritis (RA)), a female predominance is observed. However, for some autoimmune diseases, such as ankylosing spondylitis and adult-onset diabetes, there appears to be a higher risk among men. Lifestyle factors such as diet, smoking, recreational drug use, exposure to ultraviolet radiation, and environmental chemicals have all been implicated in the pathogenesis of autoimmune diseases [7, 109].

Familial aggregation and laboratory animal studies suggest a strong association between genetics and most autoimmune diseases. Genetic defects that lead to primary immunodeficiencies (PID) have identified critical steps in the process of establishing tolerance and immune regulation that are associated with clinical manifestations of autoimmunity [110, 111]. The two most common antibody deficiencies, selective IgA deficiency and common variable immunodeficiency, are associated with self-reactivity to a broad group of target tissues, and clinical manifestations of autoimmunity may appear in as many as 35% of individuals with the PID. The fact that not all individuals with a particular PID develop any or the same manifestations of autoimmunity is further support for the influence of environmental factors on the development and progression of these diseases. Susceptibility genes identified in PID have shown the importance of mutations in proteins associated with somatic recombination of T and B cell surface receptors, Fas-mediated T cell apoptosis, negative selection in the thymus, the development and activation of regulatory T cells, and the production of complement components [112]. Specific alleles within the major histocompatibility complex (MHC) gene region, functionally polymorphic genes encoding Fc and immunoinhibitory

receptors such as CTL-A4, have been implicated as genetic factors in determining disease susceptibility, pathogenesis, and the course of many autoimmune diseases.

Occupational epidemiology studies often provide the best opportunity for identifying chemical-induced modulation of the immune system in human populations, as exposure levels tend to be higher than those found outside the workplace. Work-related exposures to compounds such as crystalline silica, heavy metals, and solvents have been associated with a number of systemic autoimmune diseases. Individuals with high-level exposures to silica-containing mineral dusts have been shown to demonstrate elevated risk for a number of autoimmune diseases, including RA, scleroderma ANCA-related vasculitis, and SLE. Exposures to tobacco smoke and iron particles have been shown to increase disease incidence and exacerbate the severity of symptoms in workers exposed to silica, stressing the need to identify potentially hazardous coexposures to accurately assess the risk for development of disease.

Vinyl chloride has been linked to the development of a scleroderma-like disease characterized by skin thickening, Raynaud's phenomenon (discoloration of the extremities due to peripheral vasoconstriction), acroosteolysis (shortening of the terminal digital phalanges due to bone resorption), and pulmonary involvement. The linkage between vinyl chloride and autoimmunity stimulated research into associations between systemic autoimmune diseases and other solvents (e.g., trichloroethylene, trichloroethane, and xylenes), predominantly in occupational settings. An increased risk for systemic sclerosis was reported in several studies, but the risk is not consistent for all systemic autoimmune diseases [7].

As we better understand the consequences of immune dysregulation, there is increasing suspicion that early life exposures may lead to increased risk for autoimmune diseases later in life. A number of health concerns have been raised with regard to the children of women who received diethylstilbestrol (DES) during pregnancy to prevent preterm delivery or pregnancy loss. As part of the follow-up to a large multicenter epidemiologic study that examined the incidence of cancer and other diseases in DES-exposed and unexposed cohorts, Noller *et al.* [113] examined the self-reported prevalence of autoimmune diseases in 1,711 exposed women and 922 controls. The overall frequency of autoimmune diseases was significantly elevated in exposed women when compared with the control group (28.6 *vs.* 16.3 per thousand, $p=0.02$). A number of additional studies have suggested that the offspring of DES-treated women exhibit a variety of immune system perturbations, including enhanced T cell proliferation and elevated NK cell activity, that could contribute to immune dysregulation [114–116] and an elevated risk for autoimmune disease.

Miller *et al.* [117] have proposed a structured set of criteria to define environmentally associated autoimmune diseases in the human population. The five primary elements of these criteria are temporal plausibility; exclusion of other causative agents; dechallenge (resolution or improvement of the condition after removal of the agent); rechallenge (recurrence or worsening of the condition after reexposure to the agent); and biological plausibility. Identification of analogous cases, nearly identical cases, and evidence for a dose–response effect are also

considered as supportive of a proposed association. The proposed tiered approach provides a framework upon which to assess the level of evidence for associations between exposures to exogenous agents and autoimmune diseases. For example, the development of eosinophilia–myalgia syndrome (EMS) and toxic oil syndrome (TOS), autoimmune disorders similar to diffuse fasciitis with eosinophilia and systemic sclerosis, has been associated with the ingestion of impure L-tryptophan containing dietary supplements and the consumption of contaminated rapeseed oil produced by a particular refinery [118, 119]. A number of studies suggested that the degree of illness correlated with the amount and frequency of intake [120–122], suggesting a potential dose–response relationship. These are two of the rare instances in which there is epidemiologic evidence for a temporal association between a specific environmental exposure and the onset of autoimmunity. However, there is often a long latency period between exposure and the development of disease, and for many compounds, the weight of evidence from human studies remains only suggestive.

Animal Data

Several investigators have examined immunologic effects in inbred and autoimmune prone mouse strains following prenatal or perinatal exposure [123]. The prototypical immunotoxicant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been shown to induce thymic atrophy, alter thymocyte maturation and expression of MHC molecules, and increase the number of extrathymic autoreactive T cells [124–126], suggesting that the compound may promote autoimmunity. *In utero* exposure to TCDD has been reported to alter the time to disease onset in mice prone to autoimmune disease [127–129]. In C57Bl/6 mice, a strain not known for the development of autoimmune disease, gestational exposure to TCDD altered T cell populations in the spleen and thymus [130]. Increased immune complex and complement component 3 (C3) deposition in the glomeruli and elevated titers of autoantibodies indicate that developmental exposure to TCDD may have resulted in an increased risk for the development of autoimmunity in these mice [130].

Animal models of autoimmunity have been used to explore both molecular mechanisms and therapeutic interventions for a variety of autoimmune diseases [131]. However, there are currently no validated models to assess or identify chemicals that induce or exacerbate autoimmune diseases. The popliteal lymph node assay (PLNA), which measures nonspecific stimulation and proliferation in the lymph nodes draining chemically exposed tissues, has been shown to be a useful tool for screening for immunostimulating compounds [132]. In some situations, animal models of autoimmunity may utilize immunization with purified self-antigens, often in the presence of adjuvants, to elicit autoimmune responses. Rodents that are genetically predisposed to develop autoimmune disease, such as the lupus-prone MRL (MRL-lpr) mouse and the nonobese diabetic (NOD) mouse model for insulin-dependent diabetes, have been used to elucidate the role of specific genetic loci in the disease process. As our understanding of the genes that are associated

with predisposition for specific autoimmune diseases in the human population has increased, animal models with similar genetic defects have been used to evaluate the potential for xenobiotics to modulate autoimmunity in genetically susceptible individuals. Consistent with what is believed to occur in humans, in some of these genetically prone models, autoimmunity is induced only following exposure to chemical or biological agents.

Autoimmunity Conclusions

Although it is believed that a genetic predisposition to self-reactivity exists in all individuals with autoimmune diseases, the lack of concordance between identical twins (9–30% depending on the disease) suggests that both genetic and environmental factors interact to determine the specific disease an individual develops, and disease outcome and progression. Limited evidence from occupational and epidemiologic studies suggests associations between chemical exposures and autoimmune diseases. However, only rarely have specific cause and effect relationships between chemical exposures and autoimmunity been established. Animal models to evaluate the impact of chemicals on induction and exacerbation of autoimmunity are largely disease specific, and the lack of widely accepted screening tools has hampered our ability to predict for these types of immune perturbations and potential impact on human health.

Risk Assessment

Risk assessment for immunotoxicity should be performed using the same approaches and principles for other noncancer effects. The basic method for performing human health risk assessment for environmental exposures is a well-defined process constituting problem formulation and four evaluation steps: hazard identification, hazard characterization (or dose–response assessment), exposure assessment, and risk characterization [133–135]. Problem formulation is a planning activity in which the risk assessor defines the goals, scope, and focus of the risk assessment including factors such as at-risk populations (workers, children, *etc*.), exposure routes, temporal scope options (acute, chronic, *etc*.), and endpoints evaluated (i.e., comprehensive or a targeted assessment like immunotoxicity). The first evaluation step for developing a risk assessment, hazard identification, is the process of collecting and analyzing the full range of human and animal data for a given compound and drawing conclusions on whether or not it causes adverse effects. Dose–response assessment is the second evaluation step, with the goal of characterizing the relationship between dose and the incidence of an adverse effect. The next step, exposure assessment, details the route, nature, and extent of human contact with the compound. Risk characterization is the final step in the risk

assessment process in which the scientific evidence of adverse effects is integrated with the relevant exposure scenarios to provide a summary of the risk to human health including the assumptions and uncertainties involved in the evaluation.

Hazard Identification

Hazard identification from immunotoxicity data should result in weight of evidence (WOE) conclusions based on the available human and animal data, both positive and negative, for a given chemical. A chemical may have effects on more than one aspect of immunotoxicology, and therefore, the database should be evaluated for each type of immunotoxicity (suppression, stimulation, hypersensitivity, and autoimmunity) separately. Data from human exposures (e.g., occupational exposure studies) are preferred because fewer assumptions are required to determine the relative risk of immunotoxicity for the general population from human data than from animal data. The human data, however, are often incomplete and lack adequate exposure information or predictive immune measures. Therefore, animal data often serve as the basis for quantitative analysis to drive the risk assessment. The use of animal data for immunotoxicity risk assessment is supported by the general consistency between human evidence of immunotoxicity from clinical and epidemiological studies and the experimental animal data [2, 3, 9].

The WOE conclusions are developed on the basis of the human and experimental evidence, dose–response relationship, strength of the association, consistency of association, temporal association, coherence, specificity, analogy, and biological plausibility of the full dataset for immunotoxicity [136, 137]. Data are evaluated within the same or similar assays, as well as across divergent measures of the immune system and across multiple species. The WOE conclusions are strengthened by consistency (particularly across species, sex, or related endpoints), concordance, and breadth (range of effects) of the evidence for immunotoxicity.

Key Factors in Evaluating Immunotoxicity Across Studies

Factors such as stress, gender, species, and age may affect immunity and therefore may influence the results of immunotoxicity studies. The results themselves also must be evaluated in terms of biological significance of the observed effects. The following list discusses important considerations for evaluating data from each study for evidence of immunotoxicity.

Adversity or Biological Significance

To determine biological significance of animal data, one must consider the degree of change in a given measure of immunity that constitutes an adverse or biologically significant effect. The conservative approach is that any statistically significant

effect should be considered meaningful with larger effects receiving greater weight, provided the quality of the animal data is sufficient. This approach is based on the assumption that a linear relationship exists between loss of immune responsiveness and increased risk of developing disease. A linear relationship is consistent with our understanding of immunological processes and is supported both by animal [17] and human studies [138] in which changes in immune tests correlated progressively with increased incidence of disease over a broad range.

Mode of Action

Information on the mode of action can be used to evaluate human relevance and to help predict the types of adverse effect that might be expected to occur as well as the persistence of effects. For example, alterations in B cell function, but not T cell function, would be expected to affect resistance to intracellular pathogens, but not viral infections. Alterations in stem cells would likely have long-term effects, as compared to alterations limited to secondary lymphoid organs such as the spleen or lymph node.

Stress

It is important to control for the influence of stress on the immune system in experimental design because of the strong immunomodulatory effects of stress. If immunotoxicity is observed at dose levels which do not induce overt toxicity, the test chemical can be considered immunotoxic independent of whether it occurs via a direct effect on the immune system or an indirect effect, such as induction of a stress response. In lieu of clear evidence of general toxicity, stress-induced immunotoxicity can be determined by testing in adrenalectomized animals.

Gender Considerations

Qualitative and quantitative gender-dependent differences in baseline levels of immune function are well known in humans and laboratory animals and in part have been linked to relative levels and response to sex steroids. If data are available for both sexes, the most sensitive gender can be used for the risk assessment. For chemicals with known effects on the endocrine system, data from both sexes are preferred.

Species and Strain Considerations

In cases where species or strain differences in immunotoxicity are demonstrated, data on toxicokinetics and the mode(s) of action can help select the best animal model for immunotoxicity in humans. In the absence of data to inform selection of

the most appropriate animal model for a given chemical, the most sensitive species is utilized.

Age at Initial Exposure

The persistence of effects in adult animals has not been systematically evaluated; however, the expectation is that immune function returns to normal as immunotoxicants are cleared unless the chemical permanently damages sources of progenitor cells. On the other hand, developmental exposure has been shown to have lasting effects, persisting for weeks or months [1, 139]. Developmental immunotoxicity may be manifested qualitatively (i.e., affecting the developing immune system without affecting the adult) or quantitatively (i.e., affecting the developing immune system at lower doses). It is also suspected that the elderly may experience greater effects than young adults exposed to immunotoxic chemicals, although whether this is due to a general decline in immune responses or remodeling and dysregulation in specific cell populations remains to be determined [140].

Weighing the Evidence (WOE) to Draw Immunotoxicity Hazard Identification Conclusions

For each area of immunotoxicity (suppression, stimulation, hypersensitivity, or autoimmunity), the strength of the evidence varies by the methods used with some types of data presenting clear evidence of immunotoxicity, while the value of other data ranges from those that provide some support for immunotoxicity to equivocal.

Human Data

Epidemiological studies demonstrating an association between chemical exposure and disease burden are considered strong data, particularly if quantitative exposure information is available. Controlled clinical studies with quantitative evaluation of immune function can also provide clear evidence of immunotoxicity such as skin prick test data demonstrating hypersensitivity, autoantibodies indicating autoimmunity, or alterations in antibody responses to vaccination to support suppression or stimulation. *In vivo* functional assays to naturally occurring antigens are preferred because they test an intact immune system rather than a subset of isolated cells. However, they are not frequently performed, because *in vivo* testing procedures require injection of antigen into human subjects. The observational and *in vitro* assays most commonly used in human immunotoxicity studies (i.e., enumeration of immune system components and lymphocyte proliferation) are less invasive, but these endpoints are considered poor predictors of immunotoxicity. These less predictive immune measures can be used to support more definitive

animal and human data, to determine biological plausibility, and to consider potential mechanisms when developing the WOE conclusions for immunotoxicity.

Animal Data to Evaluate Suppression and Stimulation

The strongest support for chemical-mediated immunosuppression from animal data is provided by data demonstrating suppression in a host resistance assay. Chemical-related reduction in a functional immune assay also represents clear evidence of immunosuppression. Observational assays (lymphocyte phenotyping, cytokines, complement, *etc*.), hematology, histopathology, and organ weight data may support biological plausibility or mode of action, but these data are not generally considered to be reliable predictors of immunosuppression. Immune enhancement may be detected in assays designed to test for immunosuppression, so the database should be evaluated for stimulation after evaluating the data for suppression. Although stimulation of endpoints such as increased antibody synthesis may not necessarily constitute sufficient evidence of adversity, it should raise concern that susceptible populations may be adversely affected.

Animal Data to Evaluate Hypersensitivity

Although animal data could be used to address the risk for induction or elicitation of hypersensitivity, in practice, preventing the induction of sensitization would preclude the need to evaluate elicitation. As a result, and due to the relationship between the induction and elicitation dose, elicitation thresholds are generally not determined from animal studies. The LLNA, guinea pig maximization test, and Buehler test all present strong evidence for induction of dermal hypersensitivity or skin sensitization from animal models. Increased antigen-specific cytophilic antibodies currently represent the best measure of induction of respiratory sensitization, although there are no universally accepted animal models for respiratory sensitization. Observational assays may be useful to support biological plausibility or mode of action.

Animal Data to Evaluate Autoimmunity

Chemical-related increases in disease incidence and/or disease progression in autoimmune prone animal models are considered strong evidence of increased risk of autoimmune disease. Increased levels of autoantibodies or positive results in lymph node proliferation assays also present some evidence of autoimmunity. Observational assays, hematology, histopathology, and organ weight data are equivocal evidence but can be used to support biological plausibility or mode of action of more predictive measures of autoimmunity.

WOE Conclusions

The WOE summary for immunotoxicity hazard identification should include conclusions on the evidence for suppression, enhancement, hypersensitivity, and autoimmunity. These conclusions should describe the database in terms of consistency and biological plausibility including strengths, weakness, uncertainties, and data gaps. Modulation of the same assay in multiple species or multiple functional assays that are biologically related increases the strength of the data indicating immunotoxicity. Just as positive data on a range of assays strengthen the WOE for immunotoxicity, negative data on a range of more predictive assay such as immune function data increase confidence to support lack of immunotoxicity.

Dose–Response

Dose–response assessments can be performed for each form of immunotoxicity (suppression, unintended stimulation, hypersensitivity, and autoimmunity) supported by the database for a given chemical or simply for the lowest effect level as this would be protective of other endpoints. If dose–response evaluations are performed for multiple endpoints, factors other than the lowest effect level (e.g., the steepness of the dose–response curve and the relative severity of health effects) can be considered at later stages of the risk assessment. A dose–response relationship is a necessary criterion in demonstrating chemical immunotoxicity. The dose–response functions for immunotoxicity are generally assumed to be nonlinear and to demonstrate a threshold dose below which effects on immunity would not be expected, consistent with these assumptions for other noncancer endpoints. In addition to identifying the shape of the dose–response curve and the effective dose range for immunotoxicity, factors such as exposure (route, timing, and duration), toxicokinetics, and other issues that might affect comparisons with human exposure scenarios are identified and discussed as part of the dose–response evaluation [141, 142].

The results from dose–response analyses can be used in various ways depending on the specific goals of the risk assessment: establishment of a health-based guidance value, estimation of the margin of exposure, or quantitative estimation of the magnitude of the risk at the level of human exposure. In each case, the critical effect(s) is selected, and data from these endpoints are used to calculate the guidance values. The critical effects for immunotoxicity data should generally represent the most sensitive adverse immune endpoint(s) from the most appropriate species. Health-based guidance values can be calculated from the critical effect by dividing the dose associated with no adverse effects (the no-observed-adverse-effect-level—NOAEL, or the mathematically determined benchmark dose—BMD) by a measure of the uncertainty in the assessment (the uncertainty factors or UFs) using approaches that are generally similar across agencies [142–144].

Guidance values for suppression, unintended stimulation, and autoimmunity are calculated using all of the standard UFs (intraspecies, interspecies, database) as well as subchronic to chronic and LOAEL (lowest-observed-adverse-effect-level) to NOAEL as determined by the scope and the data used for the critical effect. Risk assessment of autoimmunity from animal data illustrates issues presented for risk assessment based on animal data that model the most sensitive subpopulation of humans. Reduction in the intraspecies UF may be considered when human risk of autoimmunity is estimated from autoimmune prone animals when an identified genetic defect allows them to be considered specific models of susceptible humans rather than the general population. UFs applied to hypersensitivity also present a special case wherein the use of an additional matrix and time and use factor has been proposed when expected human exposure differs from the experimental situation [67].

Exposure Assessment

Exposure assessment is the process of comparing the exposure levels associated with reference values obtained in the dose–response assessment to known or estimated human exposure in order to help quantify the risk to a population. Specific guidelines on exposure assessment [145, 146] and guidance specific to assessing exposure of children [147] have been published separately and will not be discussed here. The importance of route of exposure and the adequacy of route-specific animal data on immunotoxicity for a given chemical deserve particular attention for exposure assessment and are discussed below.

It is generally accepted that the circulating concentration of immunotoxicant or immunotoxic metabolite(s) determines the level of immunotoxicity, and equal concentrations of immunotoxicant in the blood following different routes of exposure should result in similar levels of systemic immunotoxicity. However, to avoid complications of distinct local toxicity, animal data on immunotoxicity should match the most likely route of human exposure for a given chemical [26, 148]. Unlike most toxicological endpoints, for immunotoxicology, the route of exposure is not simply a local increased concentration or uptake issue. There are local, partially independent portions of the immune system associated with each of the major routes of exposure: inhalation, dermal, and oral. Therefore, route of exposure is potentially more important for immunotoxicity endpoints, and since local immunotoxicological effects may occur independent of systemic immunity, a separate evaluation of local immune responses may be appropriate. For example, chemical immunotoxicity may suppress immune function at the site of exposure, such as inhalation-dependent suppression of the resident macrophage populations of the lung, without affecting immune function of macrophages elsewhere in the body [149].

Risk Characterization

Risk characterization for immunotoxicity is a synthesis of estimates of exposure levels and health risks that should be developed as for other forms of toxicity. During the summary and integration portion of the risk assessment process, the hazard identification, quantitative dose–response assessment, and exposure assessment are combined along with a critical appraisal of the toxicity information. The critical evaluation contains a review of the overall quality of the assessment including a thorough appraisal of the strengths, weaknesses, and uncertainties in the assessment as well as an evaluation of confidence in the conclusions. Risk characterization also includes a section describing risk in terms of the nature and extent of harm. Additionally, to the extent permitted by available data, risk characterization indicates how risk varies with exposure and provides information to help risk managers evaluate a range of options.

Risk Assessment Conclusions

Human health risk assessments for immunotoxicity from chemical exposure are developed in four evaluation steps: hazard identification, hazard characterization (or dose–response assessment), exposure assessment, and risk characterization. The hazard identification conclusions are developed using a weight of evidence approach based on the available human and animal data for a given chemical. The database should be evaluated separately for each type of immunotoxicity (suppression, stimulation, hypersensitivity, and autoimmunity). Dose–response relationships for immunotoxicity are expected to display a threshold dose below which effects are not observed. The possibility of route-specific, local immune responses should be considered during hazard identification and exposure assessment. Risk assessments for immunotoxicity should result in a risk characterization that summarizes the evidence for immunotoxicity risk and describes the uncertainties in the evaluation.

Conclusions

Chemical modulation of the immune system may result in suppression, enhancement, hypersensitivity, or autoimmunity. Immunosuppression associated with chemical exposure is universally regarded as adverse because immunosuppressed individuals are at greater risk for infectious or neoplastic diseases. There are well-established testing guidelines to screen and evaluate xenobiotics for the potential to cause immunosuppression. The consequences of unintended stimulation of the immune system are less clear. Nevertheless, stimulation should be considered to

be potentially adverse as it may exacerbate inflammation, hypersensitivity, or autoimmunity in susceptible populations. The most common hypersensitivity reactions to chemicals can be split into dermal effects (ACD) and respiratory effects (allergic rhinitis and asthma). There are well-established screening and testing guidelines for ACD; however, there are no standardized tests to evaluate potential respiratory sensitizers. As a result, assessment of respiratory sensitizers involves a greater reliance on human epidemiologic studies. For autoimmunity, there are a number of disease-specific animal models to evaluate xenobiotics for their potential to induce or exacerbate autoimmune diseases. However, as is the case for respiratory hypersensitivity, animal models are not available to more broadly screen chemicals for potential autoimmunity. Risk assessment for each type of immunotoxicity (suppression, stimulation, hypersensitivity, and autoimmunity) can be performed using the same general procedures as for other noncancer effects.

Acknowledgments This research was supported by the Intramural Research program of the National Institutes of Health, National Institute of Environmental Health Sciences. The findings and conclusions in this report represent the opinions of the authors and do not represent, and should not be construed to represent, statements, opinions, or conclusions of the National Toxicology Program, NIEHS, NIH, the Environmental Protection Agency, or the United States Government. This chapter has been reviewed by the Environmental Protection Agency's Office of Research and Development and approved for publication. We thank Dr. Andrew Hotchkiss and Dr. David Lehmann for their thoughtful review of this manuscript.

References

1. Luebke RW, Chen D, Dietert R, Yang Y, King M, Luster M (2006) The comparative immunotoxicity of five selected compounds following developmental or adult exposure. J Toxicol Environ Health B Crit Rev 9:1–26
2. Descotes J (2003) From clinical to human toxicology: linking animal research and risk assessment in man. Toxicol Lett 140–141:3–10
3. Vos JG, Van Loveren H (1998) Experimental studies on immunosuppression: how do they predict for man? Toxicology 129:13–26
4. van Loveren H, Cockshott A, Gebel T, Gundert-Remy U, de Jong WH, Matheson J, McGarry H, Musset L, Selgrade MK, Vickers C (2008) Skin sensitization in chemical risk assessment: report of a WHO/IPCS international workshop focusing on dose-response assessment. Regul Toxicol Pharmacol 50:155–199
5. Gerberick FG, Ryan CA, Dearman RJ, Kimber I (2007) Local lymph node assay (LLNA) for detection of sensitization capacity of chemicals. Methods 41:54–60
6. Basketter DA, Evans P, Fielder RJ, Gerberick GF, Dearman RJ, Kimber I (2002) Local lymph node assay—validation, conduct and use in practice. Food Chem Toxicol 40:593–598
7. WHO (2006) Principles and methods for assessing autoimmunity associated with exposure to chemicals, IPCS environmental health criteria 236. International programme on chemical safety. World Health Organization, Geneva, Switzerland
8. Selgrade MK (2007) Immunotoxicity: the risk is real. Toxicol Sci 100:328–332
9. Koller LD (2001) A perspective on the progression of immunotoxicology. Toxicology 160:105–110

10. Luebke RW, Parks C, Luster MI (2004) Suppression of immune function and susceptibility to infections in humans: association of immune function with clinical disease. J Immunotoxicol 1:15–24
11. Luster MI, Blanciforti LM, Germolec DR, Parks C, Kashon M, Luebke R (2004) Associating changes in the immune system with clinical diseases for interpretation in risk assessment. In: Maines M, Costa L, Reed D, Hodgson E (eds) Current protocols in toxicology. Wiley, New York, NY
12. Dietert RR (2008) Developmental immunotoxicology (DIT): windows of vulnerability, immune dysfunction and safety assessment. J Immunotoxicol 5:401–412
13. Van Loveren H, Van Amsterdam JG, Vandebriel RJ, Kimman TG, Rumke HC, Steerenberg PS, Vos JG (2001) Vaccine-induced antibody responses as parameters of the influence of endogenous and environmental factors. Environ Health Perspect 109:757–764
14. Gans H, DeHovitz R, Forghani B, Beeler J, Maldonado Y, Arvin AM (2003) Measles and mumps vaccination as a model to investigate the developing immune system: passive and active immunity during the first year of life. Vaccine 21:3398–3405
15. Van Loveren H, Germolec D, Koren H, Luster M, Nolan C, Repetto R, Smith E, Vos JG, Vogt R (1999) Report of the Bilthoven symposium: advancement of epidemiological studies in assessing the human health effects of immunotoxic agents in the environment and the workplace. Biomarkers 4:135–157
16. Luster MI, Portier C, Pait DG, White KL Jr, Gennings C, Munson AE, Rosenthal GJ (1992) Risk assessment in immunotoxicology. I. Sensitivity and predictability of immune tests. Fundam Appl Toxicol 18:200–210
17. Luster MI, Portier C, Pait DG, Rosenthal GJ, Germolec DR, Corsini E, Blaylock BL, Pollock P, Kouchi Y, Craig W, White KL, Munson AE, Comment CE (1993) Risk assessment in immunotoxicology. II. Relationships between immune and host resistance tests. Fundam Appl Toxicol 21:71–82
18. Yang EV, Glaser R (2000) Stress-induced immunomodulation: impact on immune defenses against infectious disease. Biomed Pharmacother 54:245–250
19. Glaser R, Pearson GR, Bonneau RH, Esterling BA, Atkinson C, Kiecolt-Glaser JK (1993) Stress and the memory T-cell response to the Epstein–Barr virus in healthy medical students. Health Psychol 12:435–442
20. Glaser R, Rice J, Sheridan J, Fertel R, Stout J, Speicher C, Pinsky D, Kotur M, Post A, Beck M (1987) Stress-related immune suppression: health implications. Brain Behav Immun 1:7–20
21. Esterling BA, Antoni MH, Kumar M, Schneiderman N (1993) Defensiveness, trait anxiety, and Epstein–Barr viral capsid antigen antibody titers in healthy college students. Health Psychol 12:132–139
22. Cohen S (1995) Psychological stress and susceptibility to upper respiratory infections. Am J Respir Crit Care Med 152:S53–S58
23. Biondi M, Zannino LG (1997) Psychological stress, neuroimmunomodulation, and susceptibility to infectious diseases in animals and man: a review. Psychother Psychosom 66:3–26
24. Kasl SV, Evans AS, Niederman JC (1979) Psychosocial risk factors in the developmental of infectious mononucleosis. Psychosom Med 41:445–466
25. Cohen S, Tyrrell DA, Smith AP (1991) Psychological stress and susceptibility to the common cold. N Engl J Med 325:606–612
26. WHO (1996) Principles and methods for assessing direct immunotoxicity associated with exposure to chemicals, IPCS environmental health criteria 180. International programme on chemical safety. World Health Organization, Geneva, Switzerland
27. U.S. FDA (1999) Guidance for industry: immunotoxicity testing guidance. Report. Center for Devices and Radiological Health, Rockville, MD
28. U.S. FDA (2002) Guidance for industry: immunotoxicity evaluation of investigational new drugs. Report. Center for Drug Evaluation and Research, Rockville, MD
29. Lee AN, Werth VP (2004) Activation of autoimmunity following use of immunostimulatory herbal supplements. Arch Dermatol 140:723–727

30. Holvast B, Huckriede A, Kallenberg CG, Bijl M (2007) Influenza vaccination in systemic lupus erythematosus: safe and protective? Autoimmun Rev 6:300–305
31. Elkayam O, Ablin J, Caspi D (2007) Safety and efficacy of vaccination against *Streptococcus pneumonia* in patients with rheumatic diseases. Autoimmun Rev 6:312–314
32. Gluck T, Muller-Ladner U (2008) Vaccination in patients with chronic rheumatic or autoimmune diseases. Clin Infect Dis 46:1459–1465
33. Parks CG, Conrad K, Cooper GS (1999) Occupational exposure to crystalline silica and autoimmune disease. Environ Health Perspect 107(Suppl 5):793–802
34. Sterzl I, Prochazkova J, Hrda P, Matucha P, Bartova J, Stejskal V (2006) Removal of dental amalgam decreases anti-TPO and anti-Tg autoantibodies in patients with autoimmune thyroiditis. Neuro Endocrinol Lett 27(Suppl 1):25–30
35. Gold DR, Burge HA, Carey V, Milton DK, Platts-Mills T, Weiss ST (1999) Predictors of repeated wheeze in the first year of life: the relative roles of cockroach, birth weight, acute lower respiratory illness, and maternal smoking. Am J Respir Crit Care Med 160:227–236
36. Jaakkola JJ, Gissler M (2004) Maternal smoking in pregnancy, fetal development, and childhood asthma. Am J Public Health 94:136–140
37. Ng SP, Silverstone AE, Lai ZW, Zelikoff JT (2006) Effects of prenatal exposure to cigarette smoke on offspring tumor susceptibility and associated immune mechanisms. Toxicol Sci 89:135–144
38. Weisglas-Kuperus N, Patandin S, Berbers GA, Sas TC, Mulder PG, Sauer PJ, Hooijkaas H (2000) Immunologic effects of background exposure to polychlorinated biphenyls and dioxins in Dutch preschool children. Environ Health Perspect 108:1203–1207
39. Heilmann C, Grandjean P, Weihe P, Nielsen F, Budtz-Jorgensen E (2006) Reduced antibody responses to vaccinations in children exposed to polychlorinated biphenyls. PLoS Med 3:1352–1359
40. Dallaire F, Dewailly E, Vezina C, Muckle G, Weber JP, Bruneau S, Ayotte P (2006) Effect of prenatal exposure to polychlorinated biphenyls on incidence of acute respiratory infections in preschool Inuit children. Environ Health Perspect 114:1301–1305
41. Gilmour MI, Selgrade MJK, Lambert AL (2000) Enhanced allergic sensitization in animals exposed to particulate air pollution. Inhal Toxicol 12(Suppl 3):373–380
42. Steerenberg PA, Withagen CE, van Dalen WJ, Dormans JA, Heisterkamp SH, van Loveren H, Cassee FR (2005) Dose dependency of adjuvant activity of particulate matter from five European sites in three seasons in an ovalbumin-mouse model. Inhal Toxicol 17:133–145
43. Gilmour MI, Jaakkola MS, London SJ, Nel AE, Rogers CA (2006) How exposure to environmental tobacco smoke, outdoor air pollutants, and increased pollen burdens influences the incidence of asthma. Environ Health Perspect 114:627–633
44. Selgrade MK, Illing JW, Starnes DM, Stead AG, Menache MG, Stevens MA (1988) Evaluation of effects of ozone exposure on influenza infection in mice using several indicators of susceptibility. Fundam Appl Toxicol 11:169–180
45. Teske S, Bohn AA, Hogaboam JP, Lawrence BP (2008) Aryl hydrocarbon receptor targets pathways extrinsic to bone marrow cells to enhance neutrophil recruitment during influenza virus infection. Toxicol Sci 102:89–99
46. Luebke RW, Copeland CB, Bishop LR, Daniels MJ, Gilmour MI (2002) Mortality in dioxin-exposed mice infected with influenza: mitochondrial toxicity (reye's-like syndrome) *versus* enhanced inflammation as the mode of action. Toxicol Sci 69:109–116
47. Ryan LK, Copeland LR, Daniels MJ, Costa ER, Selgrade MJ (2002) Proinflammatory and Th1 cytokine alterations following ultraviolet radiation enhancement of disease due to influenza infection in mice. Toxicol Sci 67:88–97
48. Hudson CA, Cao L, Kasten-Jolly J, Kirkwood JN, Lawrence DA (2003) Susceptibility of lupus-prone NZM mouse strains to lead exacerbation of systemic lupus erythematosus symptoms. J Toxicol Environ Health A 66:895–918

49. Rodgers KE, Imamura T, Devens BH (1986) Organophosphorus pesticide immunotoxicity: effects of *O,O,S*-trimethyl phosphorothioate on cellular and humoral immune response systems. Immunopharmacology 12:193–202
50. Rodgers KE (1997) Effects of oral administration of malathion on the course of disease in MRL-lpr mice. J Autoimmun 10:367–373
51. Selgrade MJK, Germolec DR, Luebke RW, Smialowicz RJ, Ward MD, Bowman CB (2008) Immunotoxicology. In: Smart RC, Hodgson E (eds) Molecular and biochemical toxicology. Wiley, Hoboken, NJ, pp 765–803
52. Kirchner DB (2002) The spectrum of allergic disease in the chemical industry. Int Arch Occup Environ Health 75(Suppl):S107–112
53. Treudler R, Kozovska Y, Simon JC (2008) Severe immediate type hypersensitivity reactions in 105 German adults: when to diagnose anaphylaxis. J Investig Allergol Clin Immunol 18:52–58
54. WHO (1999) Principles and methods for assessing allergic hypersensitization associated with exposure to chemicals, IPCS environmental health criteria 212. International programme on chemical safety. World Health Organization, Geneva, Switzerland
55. Friedmann PS, Moss C, Shuster S, Simpson JM (1983) Quantitative relationships between sensitizing dose of DNCB and reactivity in normal subjects. Clin Exp Immunol 53:709–715
56. Scott AE, Kashon ML, Yucesoy B, Luster MI, Tinkle SS (2002) Insights into the quantitative relationship between sensitization and challenge for allergic contact dermatitis reactions. Toxicol Appl Pharmacol 183:66–70
57. Friedmann PS (2007) The relationships between exposure dose and response in induction and elicitation of contact hypersensitivity in humans. Br J Dermatol 157:1093–1102
58. Andersen KE (2003) Occupational issues of allergic contact dermatitis. Int Arch Occup Environ Health 76:347–350
59. McNamee PM, Api AM, Basketter DA, Frank Gerberick G, Gilpin DA, Hall BM, Jowsey I, Robinson MK (2008) A review of critical factors in the conduct and interpretation of the human repeat insult patch test. Regul Toxicol Pharmacol 52:24–34
60. Kligman AM (1966) The identification of contact allergens by human assay. 3. The maximization test: a procedure for screening and rating contact sensitizers. J Invest Dermatol 47:393–409
61. Politano VT, Api AM (2008) The research institute for fragrance materials' human repeated insult patch test protocol. Regul Toxicol Pharmacol 52:35–38
62. Buehler EV (1965) Delayed contact hypersensitivity in the guinea pig. Arch Dermatol 91: 171–177
63. Magnusson B, Kligman AM (1969) The identification of contact allergens by animal assay. The guinea pig maximization test. J Invest Dermatol 52:268–276
64. Kimber I, Dearman RJ, Basketter DA, Ryan CA, Gerberick GF (2002) The local lymph node assay: past, present and future. Contact Dermatits 47:315–328
65. NIEHS (1999) The murine local lymph node assay: a test method for assessing the allergic contact dermatitis potential of chemicals/compounds. The results of an independent peer review evaluation coordinated by the Interagency Coordinating Committee for Validation of Alternative Methods (ICCVAM) and the National Toxicology Program Center for the Evaluation of Alternative Toxicological Methods (NICEATM). Report. National Toxicology Program, Research, Triangle Park, NC, pp 1–211
66. Dean JH, Twerdok LE, Tice RR, Sailstad DM, Hattan DG, Stokes WS (2001) ICCVAM evaluation of the murine local lymph node assay. Conclusions and recommendations of an independent scientific peer review panel. Regul Toxicol Pharmacol 34:258–273
67. Api AM, Basketter DA, Cadby PA, Cano MF, Ellis G, Gerberick GF, Griem P, McNamee PM, Ryan CA, Safford R (2008) Dermal sensitization quantitative risk assessment (QRA) for fragrance ingredients. Regul Toxicol Pharmacol 52:3–23
68. Griem P, Goebel C, Scheffler H (2003) Proposal for a risk assessment methodology for skin sensitization based on sensitization potency data. Regul Toxicol Pharmacol 38:269–290
69. Schneider K, Akkan Z (2004) Quantitative relationship between the local lymph node assay and human skin sensitization assays. Regul Toxicol Pharmacol 39:245–255

70. Anderson C, Hehr A, Robbins R, Hasan R, Athar M, Mukhtar H, Elmets CA (1995) Metabolic requirements for induction of contact hypersensitivity to immunotoxic polyaromatic hydrocarbons. J Immunol 155:3530–3537
71. van Och FM, Vandebriel RJ, Prinsen MK, De Jong WH, Slob W, van Loveren H (2001) Comparison of dose-responses of contact allergens using the guinea pig maximization test and the local lymph node assay. Toxicology 167:207–215
72. Yamano T, Shimizu M, Noda T (2005) Quantitative comparison of the results obtained by the multiple-dose guinea pig maximization test and the non-radioactive murine local lymph-node assay for various biocides. Toxicology 211:165–175
73. Kimber I, Basketter DA, Berthold K, Butler M, Garrigue JL, Lea L, Newsome C, Roggeband R, Steiling W, Stropp G, Waterman S, Wiemann C (2001) Skin sensitization testing in potency and risk assessment. Toxicol Sci 59:198–208
74. Devos SA, Van Der Valk PG (2002) Epicutaneous patch testing. Eur J Dermatol 12:506–513
75. Nakada T, Hostynek JJ, Maibach HI (2000) Use tests: ROAT (repeated open application test)/PUT (provocative use test): an overview. Contact Dermatits 43:1–3
76. Fischer LA, Voelund A, Andersen KE, Menne T, Johansen JD (2009) The dose-response relationship between the patch test and ROAT and the potential use for regulatory purposes. Contact Dermatits 61:201–208
77. Moorman JE, Rudd RA, Johnson CA, King M, Minor P, Bailey C, Scalia MR, Akinbami LJ (2007) National surveillance for asthma—United States, 1980-2004. MMWR Surveill Summ 56:1–54
78. Sennhauser FH, Braun-Fahrlander C, Wildhaber JH (2005) The burden of asthma in children: a European perspective. Paediatr Respir Rev 6:2–7
79. Petsonk EL (2002) Work-related asthma and implications for the general public. Environ Health Perspect 110(Suppl 4):569–572
80. Arts JH, Mommers C, de Heer C (2006) Dose-response relationships and threshold levels in skin and respiratory allergy. Crit Rev Toxicol 36:219–251
81. Merget R, Kulzer R, Dierkes-Globisch A, Breitstadt R, Gebler A, Kniffka A, Artelt S, Koenig HP, Alt F, Vormberg R, Baur X, Schultze-Werninghaus G (2000) Exposure-effect relationship of platinum salt allergy in a catalyst production plant: conclusions from a 5-year prospective cohort study. J Allergy Clin Immunol 105:364–370
82. Chung YJ, Copeland LB, Doerfler DL, Ward MD (2010) The relative allergenicity of *Stachybotrys chartarum* compared to house dust mite extracts in a mouse model. Inhal Toxicol 22:460–468
83. Sarlo K, Kirchner DB (2002) Occupational asthma and allergy in the detergent industry: new developments. Curr Opin Allergy Clin Immunol 2:97–101
84. Schweigert MK, Mackenzie DP, Sarlo K (2000) Occupational asthma and allergy associated with the use of enzymes in the detergent industry—a review of the epidemiology, toxicology and methods of prevention. Clin Exp Allergy 30:1511–1518
85. Bernstein DI, Cartier A, Cote J, Malo JL, Boulet LP, Wanner M, Milot J, L'Archeveque J, Trudeau C, Lummus Z (2002) Diisocyanate antigen-stimulated monocyte chemoattractant protein-1 synthesis has greater test efficiency than specific antibodies for identification of diisocyanate asthma. Am J Respir Crit Care Med 166:445–450
86. Bernstein JA (1996) Overview of diisocyanate occupational asthma. Toxicology 111: 181–189
87. Cartier A, Grammer L, Malo JL, Lagier F, Ghezzo H, Harris K, Patterson R (1989) Specific serum antibodies against isocyanates: association with occupational asthma. J Allergy Clin Immunol 84:507–514
88. Grammer LC, Shaughnessy MA, Henderson J, Zeiss CR, Kavich DE, Collins MJ, Pecis KM, Kenamore BD (1993) A clinical and immunologic study of workers with trimellitic-anhydride-induced immunologic lung disease after transfer to low exposure jobs. Am Rev Respir Dis 148:54–57

89. Grammer L, Shaughnessy M, Kenamore B (1998) Utility of antibody in identifying individuals who have or will develop anhydride-induced respiratory disease. Chest 114:1199–1202
90. Baur X (2003) Are we closer to developing threshold limit values for allergens in the workplace? Ann Allergy Asthma Immunol 90:11–18
91. Mapp CE, Boschetto P, Maestrelli P, Fabbri LM (2005) Occupational asthma. Am J Respir Crit Care Med 172:280–305
92. Heederik D, Houba R (2001) An exploratory quantitative risk assessment for high molecular weight sensitizers: wheat flour. Ann Occup Hyg 45:175–185
93. Sarlo K (1994) Human health risk assessment: focus on enzymes. In: Cahn A (ed) Proceedings of the 3rd World conference on detergents. American Oil Chemists Society Press, Chicago, IL, pp 54–57
94. Chung YJ, Coates NH, Viana ME, Copeland L, Vesper SJ, Selgrade MK, Ward MD (2005) Dose-dependent allergic responses to an extract of *Penicillium chrysogenum* in BALB/c mice. Toxicology 209:77–89
95. Ward MD, Chung YJ, Copeland LB, Doerfler DL (2010) A comparison of the allergic responses induced by *Penicillium chrysogenum* and house dust mite extracts in a mouse model. Indoor Air 20:380–391
96. Karol MH (1983) Concentration-dependent immunologic response to toluene diisocyanate (TDI) following inhalation exposure. Toxicol Appl Pharmacol 68:229–241
97. Instanes C, Ward MD, Hetland G (2006) The fungal biopesticide *Metarhizium anisopliae* has an adjuvant effect on the allergic response to ovalbumin in mice. Toxicol Lett 161:219–225
98. Farraj AK, Boykin E, Haykal-Coates N, Gavett SH, Doerfler D, Selgrade M (2007) Th2 cytokines in skin draining lymph nodes and serum IgE do not predict airway hypersensitivity to intranasal isocyanate exposure in mice. Toxicol Sci 100:99–108
99. Klink KJ, Meade BJ (2003) Dermal exposure to 3-amino-5-mercapto-1,2,4-triazole (AMT) induces sensitization and airway hyperreactivity in BALB/c mice. Toxicol Sci 75:89–98
100. Sailstad DM, Ward MD, Boykin EH, Selgrade MK (2003) A murine model for low molecular weight chemicals: differentiation of respiratory sensitizers (TMA) from contact sensitizers (DNFB). Toxicology 194:147–161
101. Yokota K, Johyama Y, Yamaguchi K, Takeshita T, Morimoto K (1999) Exposure-response relationships in rhinitis and conjunctivitis caused by methyltetrahydrophthalic anhydride. Int Arch Occup Environ Health 72:14–18
102. Baur X, Chen Z, Liebers V (1998) Exposure-response relationships of occupational inhalative allergens. Clin Exp Allergy 28:537–544
103. Pauluhn J, Mohr U (1994) Assessment of respiratory hypersensitivity in guinea-pigs sensitized to diphenylmethane-4,4′-diisocyanate (MDI) and challenged with MDI, acetylcholine or MDI-albumin conjugate. Toxicology 92:53–74
104. Botham PA, Rattray NJ, Woodcock DR, Walsh ST, Hext PM (1989) The induction of respiratory allergy in guinea-pigs following intradermal injection of trimellitic anhydride: a comparison with the response to 2,4-dinitrochlorobenzene. Toxicol Lett 47:25–39
105. Arts JH, Kuper CF, Spoor SM, Bloksma N (1998) Airway morphology and function of rats following dermal sensitization and respiratory challenge with low molecular weight chemicals. Toxicol Appl Pharmacol 152:66–76
106. Zhang XD, Fedan JS, Lewis DM, Siegel PD (2004) Asthmalike biphasic airway responses in Brown Norway rats sensitized by dermal exposure to dry trimellitic anhydride powder. J Allergy Clin Immunol 113:320–326
107. Arts JH, Muijser H, Appel MJ, Frieke Kuper C, Bessems JG, Woutersen RA (2004) Subacute (28-day) toxicity of furfural in Fischer 344 rats: a comparison of the oral and inhalation route. Food Chem Toxicol 42:1389–1399
108. Pollard KM, Hultman P, Kono DH (2010) Toxicology of autoimmune diseases. Chem Res Toxicol 23:455–466
109. Heindel J, Cooper GS, Germolec D, Selgrade M (1999) Linking environmental agents to autoimmune diseases. Environ Health Perspect 107(Suppl 5):659–813

110. Carneiro-Sampaio M, Coutinho A (2007) Tolerance and autoimmunity: lessons at the bedside of primary immunodeficiencies. Adv Immunol 95:51–82
111. Torgerson TR (2008) Immune dysregulation in primary immunodeficiency disorders. Immunol Allergy Clin North Am 28:315–327
112. Rose NR, Mackay IR (2006) The autoimmune diseases. Elsevier, St. Louis, MO
113. Noller KL, Blair PB, O'Brien PC, Melton LJ 3rd, Offord JR, Kaufman RH, Colton T (1988) Increased occurrence of autoimmune disease among women exposed *in utero* to diethylstilbestrol. Fertil Steril 49:1080–1082
114. Ford CD, Johnson GH, Smith WG (1983) Natural killer cells in *in utero* diethylstilbesterol-exposed patients. Gynecol Oncol 16:400–404
115. Ways SC, Mortola JF, Zvaifler NJ, Weiss RJ, Yen SS (1987) Alterations in immune responsiveness in women exposed to diethylstilbestrol *in utero*. Fertil Steril 48:193–197
116. Burke L, Segall-Blank M, Lorenzo C, Dynesius-Trentham R, Trentham D, Mortola JF (2001) Altered immune response in adult women exposed to diethylstilbestrol *in utero*. Am J Obstet Gynecol 185:78–81
117. Miller FW, Hess EV, Clauw DJ, Hertzman PA, Pincus T, Silver RM, Mayes MD, Varga J, Medsger TA Jr, Love LA (2000) Approaches for identifying and defining environmentally associated rheumatic disorders. Arthritis Rheum 43:243–249
118. Patterson R, Germolec D (2005) Toxic oil syndrome: review of immune aspects of the disease. J Immunotoxicol 2:51–58
119. Kaufman LD, Krupp LB (1995) Eosinophilia-myalgia syndrome, toxic-oil syndrome, and diffuse fasciitis with eosinophilia. Curr Opin Rheumatol 7:560–567
120. Back EE, Henning KJ, Kallenbach LR, Brix KA, Gunn RA, Melius JM (1993) Risk factors for developing eosinophilia myalgia syndrome among L-tryptophan users in New York. J Rheumatol 20:666–672
121. Kamb ML, Murphy JJ, Jones JL, Caston JC, Nederlof K, Horney LF, Swygert LA, Falk H, Kilbourne EM (1992) Eosinophilia-myalgia syndrome in L-tryptophan-exposed patients. JAMA 267:77–82
122. Tabuenca JM (1981) Toxic-allergic syndrome caused by ingestion of rapeseed oil denatured with aniline. Lancet 2:567–568
123. Holladay SD (1999) Prenatal immunotoxicant exposure and postnatal autoimmune disease. Environ Health Perspect 107(Suppl 5):687–691
124. Holladay SD, Lindstrom P, Blaylock BL, Comment CE, Germolec DR, Heindell JJ, Luster MI (1991) Perinatal thymocyte antigen expression and postnatal immune development altered by gestational exposure to tetrachlorodibenzo-*p*-dioxin (TCDD). Teratology 44:385–393
125. Blaylock BL, Holladay SD, Comment CE, Heindel JJ, Luster MI (1992) Exposure to tetrachlorodibenzo-*p*-dioxin (TCDD) alters fetal thymocyte maturation. Toxicol Appl Pharmacol 112:207–213
126. Silverstone AE, Frazier DE, Fiore NC, Soults JA, Gasiewicz TA (1994) Dexamethasone, β-estradiol, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin elicit thymic atrophy through different cellular targets. Toxicol Appl Pharmacol 126:248–259
127. Mustafa A, Holladay SD, Goff M, Witonsky S, Kerr R, Weinstein DA, Karpuzoglu-Belgin E, Gogal RM Jr (2009) Developmental exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin alters postnatal T cell phenotypes and T cell function and exacerbates autoimmune lupus in 24-week-old SNF1 mice. Birth Defects Res A Clin Mol Teratol 85:828–836
128. Silverstone AE, Gavalchin J, Gasiewicz TA (1998) TCDD, DES, and estradiol potentiate a lupus-like autoimmune nephritis in NZB × SWR (SNF_1) mice. Toxicologist 42:403
129. Smith DA, Germolec DR (2000) Developmental exposure to TCDD and mercuric chloride in autoimmune-prone MRL/lpr mice. Toxicologist 54:8
130. Mustafa A, Holladay SD, Goff M, Witonsky SG, Kerr R, Reilly CM, Sponenberg DP, Gogal RM Jr (2008) An enhanced postnatal autoimmune profile in 24 week-old C57BL/6 mice developmentally exposed to TCDD. Toxicol Appl Pharmacol 232:51–59
131. Germolec DR (2005) Autoimmune diseases, animal models. In: Vohr HW (ed) Encyclopedic reference of immunotoxicology. Springer, Berlin, Germany, pp 75–79

132. Pieters R, Ezendam J, Bleumink R, Bol M, Nierkens S (2002) Predictive testing for autoimmunity. Toxicol Lett 127:83–91
133. NRC (1983) Risk assessment in the federal government: managing the process, Committee on the institutional means for assessment of risks to public health, Commission on life sciences. National Research Council, Washington DC, pp 1–192
134. NRC (1994) Science and judgment in risk assessment, Committee on risk assessment of hazardous air pollutants, Board on environmental studies and toxicology, Commission on life sciences. National Research Council, Washington DC, pp 1–672
135. WHO (1999) Principles for the assessment of risks to human health from exposure to chemicals, IPCS environmental health criteria 210. International programme on chemical safety. World Health Organization, Geneva, Switzerland
136. Hill AB (1965) The environment and disease: association or causation? Proc R Soc Med 58:295–300
137. Weed DL (2005) Weight of evidence: a review of concept and methods. Risk Analysis 25:1545–1557
138. Luster MI, Germolec DR, Parks C, Blanciforti L, Kashon M, Luebke RW (2005) Associating changes in the immune system with clinical diseases for interpretation in risk assessment. In: Tryphonas H, Fournier M, Blakley BR, Smits JE, Brousseau P (eds) Investigative immunotoxicology. Taylor & Francis, New York, NY, pp 165–182
139. Dietert RR, Dietert JM (2007) Early-life immune insult and developmental immunotoxicity (DIT)-associated diseases: potential of herbal- and fungal-derived medicinals. Curr Med Chem 14:1075–1085
140. Huang H, Patel D, Manton K (2005) The immune system in aging: roles of cytokines, T cells and NK cells. Front Biosci 10:192–215
141. U.S. EPA (2002) A review of the reference dose and reference concentration processes. Report. Risk Assessment Forum, Washington, DC, pp 1–192
142. WHO (2009) Principles for modelling dose-response for the risk assessment of chemicals, IPCS environmental health criteria 239. International programme on chemical safety. World Health Organization, Geneva, Switzerland
143. U.S. EPA (1995) Use of the benchmark dose approach in health risk assessment. Report. Risk Assessment Forum, Washington DC, pp 1–93
144. U.S. EPA (2000) Benchmark dose technical guidance document. External review draft. Report. Risk Assessment Forum, Washington DC, pp 1–96
145. WHO (2000) Human exposure assessment, IPCS environmental health criteria 214. International programme on chemical safety. World Health Organization, Geneva, Switzerland
146. U.S. EPA (1992) Guidelines for exposure assessment. Report. Risk Assessment Forum, Washington DC, pp 1–126
147. U.S. EPA (2005) Guidance on selecting age groups for monitoring and assessing childhood exposures to environmental contaminants. Report. Risk Assessment Forum, Washington DC, pp 1–50
148. U.S. EPA (1998) Health effects test guidelines: OPPTS 870.7800 immunotoxicity. Report. Office of Prevention, Pesticides and Toxic Substances, Washington DC
149. Selgrade MK, Gilmour MI (2006) Immunotoxicology of inhaled compounds—assessing risks of local immune suppression and hypersensitivity. J Toxicol Environ Health A 69: 827–844

Chemical Sensitization and Allergotoxicology

Rosette L. Van Den Heuvel, Nathalie Lambrechts, Sandra Verstraelen, Inge C. Nelissen, and Greet E.R. Schoeters

Abstract Chemical sensitization remains an important environmental and occupational health issue. A wide range of substances have been shown to possess the ability to induce skin sensitization or respiratory sensitization. As a consequence, there is a need to have appropriate methods to identify sensitizing agents. Although a considerable investment has been made in exploring opportunities to develop methods for hazard identification and characterization, there are, as yet, no validated nonanimal methods available. A state of the art of the different *in vitro* approaches to identify contact and respiratory capacity of chemicals is covered in this chapter.

Keywords Gene biomarkers · *In chemico* models · *In vitro* models · *In silico* models · Mechanistic understanding · Potency · Respiratory sensitization · Sensitization · Skin sensitization

Introduction

Chemical-induced sensitization is the induction of the allergic immune response following exposure to a chemical. Allergic reactions to chemicals predominantly manifest adverse effects in the skin and the respiratory tract. Skin sensitization results in allergic contact dermatitis, and respiratory sensitization causes allergic

R.L. Van Den Heuvel (✉), N. Lambrechts, S. Verstraelen, I.C. Nelissen, and G.E.R. Schoeters
Environmental Risk and Health Unit—Toxicology, Flemish Institute for Technological Research (VITO N.V.), Centre for Advanced R&D on Alternative Methods (CARDAM), Boeretang 200, 2400 Mol, Belgium
e-mail: rosette.vandenheuvel@vito.be

A. Luch (ed.), *Molecular, Clinical and Environmental Toxicology*,
Experientia Supplementum 101, DOI 10.1007/978-3-7643-8340-4_10,

rhinitis and asthma. Following topical exposure, contact sensitization is the usual outcome. The skin is also the target organ following oral exposure. A major route of exposure that is of concern for respiratory allergies is inhalation, but the skin and the gastrointestinal route may also be of importance.

Allergotoxicology studies the influence of pollutants on the development of allergic reactions and diseases [1]. Interaction between air pollutants and allergens can take place both outside and inside the exposed person and, as a consequence, influence allergic diseases. Pollutants may be the carriers of allergens and may exacerbate allergic reactions and diseases [2].

The health problems of allergic diseases together with the continuous introduction of new chemicals to workers and consumers make the identification of agents with a high likelihood to induce allergic sensitization a challenging area in immunotoxicology. The European REACH (Registration, Evaluation, Authorization and restriction of Chemicals) legislation [3] and the 7th Amendment to the Cosmetics Directive banning *in vivo* testing for cosmetics products and their ingredients [4] emphasize the need for nonanimal alternative test methods and testing strategies. Moreover, the vision of toxicity testing in the twenty-first century of the National Research Council (NRC) [5] describes a paradigm shift in toxicity testing and risk assessment that focuses on "toxicity pathways." The goal is to determine how exposure to environmental/occupational agents can perturb molecular pathways that maintain cell function, causing a cascade of subsequent events leading to adverse health effects. The envisaged process includes chemical characterization, target-specific and mechanism-based toxicity testing, and dose–response and extrapolation modeling based on population-based and human exposure data for translating cellular tests to whole human systems. Where animal experiments used to be the most important approach for toxicity testing, the future is seen in the strength of quantitative high-throughput *in vitro* and *in silico* approaches based on human material and advances in toxicogenomics, bioinformatics, systems biology, epigenetics, and computational toxicology (e.g., (Q)SAR) [6].

Nonanimal Approaches for Sensitization Testing

Non-Cell-Based Approaches to Assess Sensitization

In line with applicable legislations and the above-described altered toxicology vision, considerable efforts are being performed to accurately identify and characterize skin and/or respiratory sensitizing chemicals without the need for animal experimentation. One can identify three main strategies (*in silico–in chemico–in vitro*) that each show limitations and inconsistencies, making them not suitable for use as single predictors for sensitizing properties [7–9]. Since sensitization is a multistage toxicological process, each of these strategies may

represent different stages and/or chemical classes and be ultimately combined in an integrated approach.

For the identification of skin and respiratory allergens, the use of *in silico* approaches has made its entrance in the last decade. These approaches identify allergens using (quantitative) structure activity relationships ((Q)SAR) methods, which involve a combination of computational chemistry, biology, and statistics [10–13]. (Q)SAR have been used to correlate chemical function with chemical structure, on the assumption that chemical structure is a determinant of reactive properties and hence biological activity. *In silico* models should be built on a good set of empirical data of adequate chemical coverage. The approach to estimate the skin sensitization potential of a chemical *in silico* is based on the concept that the ability of chemicals (haptens) to react with proteins to form covalently linked conjugates, based on electrophilic characteristics in their structure, is correlated with their skin sensitization capability [14]. For skin sensitization, the available published QSAR models fall into either a mechanism-based category or are empirically derived using statistical approaches (global models). Where the former is developed for small sets of structurally similar chemicals [15], the latter is often based on large datasets of diverse structures without an attempt to rationalize the underlying sensitization mechanism [16]. Because of the uncertainties regarding the molecular mechanisms for low molecular weight (LMW) respiratory sensitizers, (Q)SAR seem particularly appropriate because they make no a priori mechanistic assumptions [17].

Additionally, non-cell-based *in chemico* approaches are being explored as alternatives to animal models, including the assessment of chemical–protein reactivity. Like skin allergens, respiratory chemical allergens or their metabolites have electrophilic properties and need to react with nucleophilic amino acids in skin- or lung-derived proteins for the effective induction of sensitization. An *in chemico* quantitative peptide reactivity assay based on lysine and cysteine, which are most often cited, is under development for screening chemical skin sensitizers, and this assay may also be used for testing respiratory chemical sensitizers to assess whether their reactivity with peptides is different from chemical skin allergens [14, 18–23]. One important consideration in this approach is that many chemicals exist as prohaptens, which require metabolic conversion to become protein reactive. Therefore, such assays should consider incorporation of a metabolic activation step [24].

In Vitro Cell-Based Models to Assess Sensitization

As skin and respiratory sensitizations are complex immunological disorders, many cell types are involved in their pathogenesis and each undergoes significant changes during these processes. In developing a screening assay for assessment of a chemical's sensitization potential based on *in vitro* technology, the choice of appropriate cell types and mechanism-based endpoints is therefore important. Different experimental approaches have been used to understand the underlying mechanisms and to

assess the potential hazardous effects of LMW industrial compounds. While such *in vitro* methods fully represent an implementation of the vision on toxicity testing in the twenty-first century, there are also challenges to be identified and obstacles to be overcome. The reliance on *in vitro* methods present the long-debated problems with this approach, including the role of metabolism, the ability to extrapolate *in vitro* concentrations to relevant *in vivo* doses, the ability to understand organ interactions, the ability to detect epigenetic changes and other unknown mechanisms, and the fact that cell lines may have some abnormal biology [25].

Various cell types are involved in skin allergy and respiratory allergy, among which epithelial cells, dendritic cells (DC), and macrophages are the most important players in the sensitization phase [26, 27], and are therefore a good starting point for the development of such a test system. An adequate *in vitro* model should be simple, reproducible, quantifiable, relevant for the human situation, amenable to validate, and accepted by industry. Primary cells derived from human skin or lung are relevant cells in this context because they mostly resemble the real-life situation. The disadvantages of such cell systems are donor variability, and they are difficult to obtain in sufficient number and to maintain. In contrast, immortalized cell lines represent valuable models because of their high availability, reproducibility, and standardization potential. A cell line originates from a single donor, and therefore, donor-to-donor variation is not applicable. However, it should be noted that variations can be introduced within a cell line by deviating from the standard protocol or by culturing the cells at high passage number. Because most of them are readily available from various cell and tissue companies, large numbers of cells can be grown in a short time.

Many of the single cell-based *in vitro* assays will only be able to assess one aspect of the multistep process required to achieve sensitization. Recently, progress has been made in combining various cell types into coculture and *ex vivo* models (e.g., precision-cut lung slices) [28–30], which is a good step forward to an *in vivo*-like situation. Also air-lifted, three-dimensional human skin epidermis and lung epithelium models have been reconstructed and are being tested for their capacity to discriminate sensitizing from nonsensitizing chemicals [31–33]. These models are fully in their infancy and additional research is necessary.

Besides the choice of an appropriate cell model, it is also important to study relevant endpoints in the context of skin and respiratory sensitization. New tests should be based on the mode of action of chemicals and should focus on toxicity pathways, as proposed by the NRC. For this purpose, transcriptomics, proteomics, and metabolomics are promising tools that may provide more sensitive, mechanism-based biomarkers. For epithelial cells, DC, and macrophages, gene expression profiles derived in global gene expression studies may shed a light on the pathways that are affected by immunotoxic compounds in general and immunotoxic compounds of different potency in particular. In contrast to classical cellular endpoints which mostly have a limited dynamic range, highly specific biomarkers may be inferred using signature expression profiles that are able to classify chemicals according to their sensitizing potential and thus aid in the hazard identification of existing and novel chemical compounds.

Another question to be solved is the possibility to distinguish between contact and respiratory sensitizers. Given the general similarity of the sensitization response in skin and respiratory allergy, it has been suggested that the advances made in the safety assessment of skin sensitization might be used in concert with, or as the basis for, novel approaches for the assessment of respiratory sensitization potential. However, due to the known mechanistic differences and the more serious health and regulatory implications for classification as a respiratory allergen, the accurate identification of these compounds and their distinction from compounds inducing contact allergy is critical. The use of "omics" tools might help in this context.

A state of the art of the different *in vitro* human cell-based approaches to identify contact and respiratory sensitizing capacity, which among other alternative methods (*in silico, in chemico*) are proposed as part of an integrated testing system [34], is described below. For a comprehensive overview, the reader is referred to [8, 9, 31, 35, 36].

Skin Sensitization

Allergic contact dermatitis (ACD) is the manifestation of an allergic response in susceptible individuals caused by skin contact with an allergen and is the most prevalent form of immunotoxicity found in humans. Clinical symptoms of ACD include red rash, blisters or wheals, and/or itchy, burning skin usually confined to the area of allergen contact. The origin and nature of compounds able to induce ACD is very diverse, but all share some common features that govern skin penetration and uptake by immunogenic cells. The first barrier a chemical must cross in order to behave as a chemical skin sensitizer is the *stratum corneum*. To facilitate penetration through the viable layers of the outermost part of the skin, the epidermis, chemicals, generally denoted as haptens, should be hydrophobic (Log $K_{o/w}$ values in the ranges of -3 to 5) and small (molecular weights less than 500 Da) [37, 38]. Since these molecules are too small to be antigenic themselves, haptens readily conjugate to epidermal and dermal molecules. These then act as carrier proteins to form the hapten–carrier complex that finally acts as the antigen. Most haptens bear electrophilic residues which account for covalent bonds to the nucleophilic residues of cutaneous proteins [26, 39]. Of note is that not all sensitizers generate covalent interaction, e.g., metal ions interact via protein–metal chelate complexes, and recent evidence suggests that noncovalent interactions with the major histocompatibility complex (MHC) constitute a novel type of hapten epitope [40]. Haptens often derive from unstable chemicals, the so-called prohaptens, which need an additional metabolic activation step *in vivo* in the epidermis to be converted into an electrophilic hapten endowed with antigenic properties [41].

ACD is a form of delayed-type hypersensitivity (DTH) reaction and is dependent upon cell-mediated immune function and the activity of T lymphocytes [26, 42, 43].

The pathophysiology of ACD consists of two distinct phases: the sensitization phase also referred to as the induction phase and the elicitation phase.

Following first encounter with the chemical sensitizer, during the so-called *induction phase*, a pattern of changes is provoked in the skin to ensure that the immune system is sensitized for an allergic response. Epidermal keratinocytes (KC) constitute an important player in the induction and maintenance of inflammatory cells within the skin through the production of a variety of immunological active cytokines/factors and expression of HLA molecules and cell adhesion molecules. Cutaneous dendritic cells, represented by Langerhans cells (LC) forming a contiguous network within the epidermis [44] and the dermis [45], constitute the true initiators and modulators of immune responses [46]. They form a system of antigen-presenting cells (APC) which exhibit all accessory cell functions, i.e., high expression of MHC (major histocompatibility complex) molecules mediating antigen presentation, as well as of cellular adhesion and costimulatory molecules [47, 48]. LC reside as relatively immature DC characterized by a high capacity to internalize and process skin-encountered antigen. Following antigen uptake, APC become activated and migrate via the lymphatic system to the regional lymph nodes (LN) where they present antigens to T cells [49]. Upon activation, LC upregulate the expression of specific surface molecules (e.g., MHC), adhesion molecules (e.g., CD54), costimulatory molecules (e.g., CD80, CD86), cytokines, and changes in chemokine receptor levels (e.g., CCR1, CCR4) [50–52]. These alterations of the cell's appearance are referred to as the DC maturation process. Following skin contact with the antigen, well-characterized molecular events regulate the interplay between KC and LC. IL-1β [53], TNF-α, and GM-CSF [54, 55] facilitate migration of LC toward the lymph nodes [56]. Along with their migration and settling within the draining lymph nodes, they adopt a strongly veiled, interdigitating appearance, thus maximizing the chances of productive encounters with naïve T lymphocytes. To acquire clonal expansion of T cells, three distinct signals induce their activation. These include triggering of T-cell receptor molecules (TCR) by MHC molecules, costimulation directed by increased expression of costimulatory molecules on the surface of LC, and stimulation by several cytokines [40]. Upon activation, allergen-specific T cells start secreting IL-2, a potent T-cell growth-promoting factor, and thereby inducing abundant T-cell proliferation [57]. The progeny of primed T cells subsequently sets free via efferent lymphatic vessels and the thoracic duct into the blood [58]. Memory T cells can again leave the circulation and go into lymphoid organs anywhere in the body, thus rapidly ensuring systemic memory.

Once sensitized, individuals can develop ACD with manifestation of clinical symptoms (erythema and induration with a climax within 2–3 days) upon reexposure to the contact allergen during the so-called elicitation phase. After renewed contact, the antigen is taken up by APC as in the sensitization phase which then quickly migrate into regional lymphoid tissue and en route present antigen to sensitized memory T cells within 4–8 h. Cytokines such as IL-1β and TNF-β, as well as chemokines, are released by activated keratinocytes, leading to the recruitment of antigen-specific memory T cells from the blood. Subsequently, infiltrating cells consisting of monocytes, DC, and both $CD4^+$ and $CD8^+$ T cells enter the

epidermis and dermis and further amplify the inflammatory response by the production of chemokines and cytokines [45, 59].

In Vitro Assays to Assess Skin Sensitization Hazard

The development of a screening assay based on *in vitro* technology is perhaps the most intensively investigated field in skin sensitization toxicology. Below, different cell types studied for their capacity to distinguish skin sensitizers from nonsensitizing substances are outlined together with the various endpoints that are looked at.

At the Epithelial Barrier

KC are the predominant cell type in the skin and the first cells encountered by chemical exposure. Although their potential to produce discriminating cytokine profiles and oxidative stress responses in KC models, such as the human keratinocyte cell line HaCaT, upon skin sensitizing exposure has been described, the number of studies to develop predictive assays is still confined [60–63]. In general, it seems that studies with keratinocytes prove to be helpful in understanding the immune response to sensitizers and irritants but that the response is rather nonspecific. It looks like keratinocytes signal "danger" and in this way activate different elements of the immune system [64, 65]. Yet, recently, it has been established that primary KC and HaCaT may add significant value to an *in vitro* model for screening skin sensitizers as they allow assessment of the qualitative contribution of epidermal phase I/phase II enzymes involved in xenobiotic metabolism [66].

The Dendritic Cell

Due to their central role during the induction process, DC were rapidly considered as an obvious opportunity for developing *in vitro* approaches for the detection of potential skin sensitizers [67]. Obtaining a sufficient number of human DC from skin to be routinely used in screening assays for skin sensitization remains extremely difficult. Advances in the *in vitro* generation of dendritic-like cells from human sources have led to valuable alternatives, mimicking *in vivo* skin DC. Two main primary-derived DC-like cell models exist, generated either from human peripheral $CD14^+$ blood monocytes (CD14-DC) or from cord blood or bone marrow-derived $CD34^+$ hematopoietic precursors (CD34-DC) [68, 69]. In order to circumvent the limitation of donor variability, researchers have explored the use of DC-like cell lines as *in vitro* predictive models for skin sensitization [70]. A number of human dendritic-like cell lines have been investigated, including the human myeloid cell lines KG-1 [71], THP-1 [72, 73], U937 [74], and MUTZ-3 [75].

One of the earliest and most extensively studied endpoints to identify sensitizers has been the altered expression of cell surface markers on DC during their maturation. Currently, well-known DC cell surface maturation markers are being scrutinized, and significant expression changes of HLA-DR, CD80, CD83, CD86, CD54, and CCR7 have been reported frequently in both primary DC and DC-like cell lines [67, 70, 76–89]. Of all these possible biomarkers, increased CD86 expression has been examined most extensively and appears to be most promising. The human Cell Line Activation Test (h-CLAT) is a promising alternative method and examines the level of CD86 and CD54 expression on the surface of THP-1 cells, a human monocyte leukemia cell line [90]. The h-CLAT has been evaluated by five independent laboratories in several ring trials coordinated by the European Cosmetics Association (COLIPA). The overall accuracy of 84% indicated a good prediction performance of the h-CLAT [91].

Upon DC maturation, changes in cytokine secretion also occur. To this end, multiple studies have been conducted in which the production of cytokines was explored as possible endpoint in an *in vitro* assay to identify chemical skin sensitizers. The most extensively studied cytokines and chemokines in DC-based assays are IL-1β, IL-12, IL-18, TNF-α, CXCL8, and CXCL12 [67, 78, 80, 81, 84, 85, 89, 92–101]. Despite the numerous attempts, studies based on the expression of surface markers and cytokines have indicated that their response patterns may be chemical dependent and therefore do not allow the identification of all sensitizers and that they have a limited dynamic range [102].

As a more recent approach, transcript profiling has been applied for detecting genomic fingerprints that are induced in both monocyte-derived dendritic cells (Mo-DC) and CD34-DC models by a single or a group of chemical sensitizers, leading to potential novel and unique biomarkers of interaction of primary DC with sensitizers [103–108]. High-density microarray analyses performed in Mo-DC after treatment with the sensitizer dinitrobenzene sulfonic acid allowed to derive a list of potential target genes [107], whereas similar analyses performed on CD34-DC exposed to our known sensitizers and two irritants revealed 119 significantly discriminating genes [109]. In both Mo-DC and CD34-DC biomarkers identified by transcriptomics, analyses were further confirmed in an expanded chemical test set by performing real-time RT-PCR [110, 111]. In the CD34-DC model, these experiments have led to the development of a prediction model (VITOSENS®) for skin sensitization potential based on the differential response of C-C chemokine receptor 2 (*CCR2*) and cAMP-responsive element modulator (*CREM*) to an expanded set of 21 (non)-sensitizing chemicals (Fig. 1). So far, this primary DC-based alternative test showed a high predictive power with a concordance of 89%, specificity of 97%, and sensitivity of 82% at the level of donor samples [111]. A recent study indicated that in the THP-1 cell line, the expression pattern of biomarkers identified in primary CD34-DC appeared to be less discriminating toward (non-)sensitizing exposure [112].

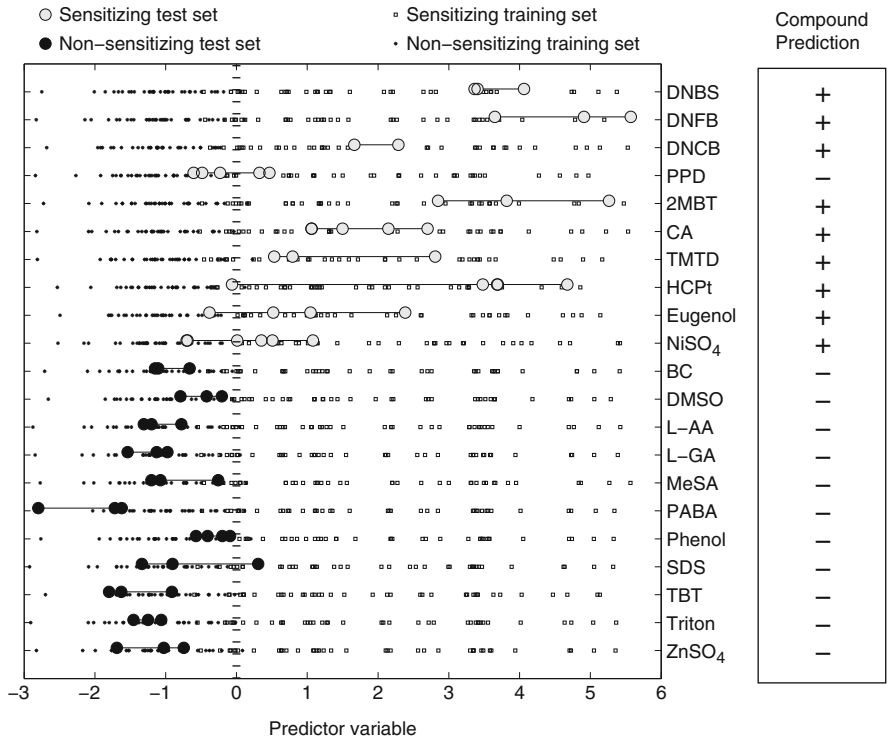


Fig. 1 Comparison predictions of the VITOSENS® model in a cross-validation. Per test compound, each *circle* corresponds to a donor sample and is represented by its predictor value: positive = sensitized, negative = nonsensitized. The *right panel* gives the prediction per compound by applying a majority rule on the representing test samples: + = sensitizer, − = nonsensitizer. Abbreviations: *DNBS* dinitrobenzenesulfonic acid, *DNFB* dinitrofluorobenzene, *DNCB* dinitrochlorobenzene, *PPD* *p*-phenylenediamine, *2-MBT* 2-mercaptobenzothiazole, *CA* cinnamaldehyde, *TMTD* tetramethylthiuram disulfide, *HCPt* ammonium hexachloroplatinate IV, *Eug* eugenol, *$NiSO_4$* nickel sulfate, *BC* benzalkonium chloride, *DMSO* dimethylsulfoxide, *L-AA* L-ascorbic acid, *L-GA* L-glutamic acid, *MeSA* methyl salicylate, *PAPA* *p*-aminobenzoic acid, *SDS* sodium lauryl/dodecyl sulfate, *TBT* tributyltin chloride, *Triton* Triton X-100, *$ZnSO_4$* zinc sulfate

T-Cell Assays

The activation and clonal expansion of allergen-responsive T cells represents the central event in the acquisition of skin sensitization. A relevant target for *in vitro* screening of skin sensitizers would therefore be detecting the specific response among naïve T lymphocytes as provoked by skin-sensitizing chemicals. One research group has reported discrimination of a first selection of sensitizers from nonsensitizers based on the induction of T-cell proliferation after exposure of cocultures of CD14-DC and autologous T cells [113]. To date however, no progress was reported from such assays. The challenge in this method lies in the identification

of what is likely to be a weak signal against a rather noisy background and the interference of considerable donor variation. Recently, a novel optimization protocol for the induction of T cells has been published which is free of exogenous growth factors [114]. This assay which is developed in the context of vaccine evaluation could be an opportunity for developing the first *in vitro* assay to identify skin sensitizers based on T-cell stimulation.

Coculture and 3D Models

Coculture systems and more sophisticated models created in order to reconstruct the typical skin layers found *in vivo* are gaining interest in the development of screening assays for skin-sensitizing chemicals.

Besides the model integrating LCs derived from $CD34^+$ progenitor cells into reconstructed human epidermis [115, 116], another example that was published recently is the "loose-fit coculture-based sensitization assay" (LCSA). This model is composed of a single layer of primary human KC and of allogenic free-floating monocytes, which differentiate to DC-like cells in the presence of exogenous cytokines [117, 118]. Based on the stimulation of CD86 expression and cell viability of DC after exposure, the skin-sensitizing potential of chemicals could be estimated [30]. Such immunocompetent tools are currently being developed in a larger European Commission-sponsored integrated Framework 6 project (Sens-it-iv: http://www.sens-it-iv.eu).

Today, several commercial providers propose reconstructed skin or epidermis, which have been validated as *in vitro* assays for skin irritation, e.g., the EpiDerm™ skin model (MatTek, US) and the EPISKIN™ reconstituted human epidermis model (RHE) (SkinEthic, L'Oréal, France) [119]. *In vitro* assays for chemical sensitization based on these 3D modes are still under development [120]. Levels of extracellular IL-1α and IL-8 in the RHE have been studied for the prediction of skin irritation and/or sensitization [121].

Mechanistic Role of Skin Sensitization Biomarkers

Although a lot of promising *in vitro* assays are being developed, it should always be kept in mind that since the desire is to have a test system that incorporates an understanding of the biological mechanisms of the induction of skin sensitization, it will be necessary and appropriate to consider the biological relevance of selected candidate genes [122]. Linking the identified biomarkers as functional players in the complex process of skin sensitization will emphasize the relevance of the *in vitro* assay for screening possible human skin-sensitizing chemicals.

When an *in vitro* assay incorporates the complex, underlying molecular events of skin sensitization, it will aid in a better extrapolation of data and an improvement of risk assessment. Therefore, linking assay markers as functional players in the

complex process of skin sensitization will emphasize the relevance of the *in vitro* test for screening possible human skin-sensitizing chemicals.

One such an example is the biological characterization of marker genes in the VITOSENS® assay [123]. Using pathway analysis software, a literature-based network was constructed around VITOSENS® markers, in which the presence of other skin sensitization-related molecules was revealed. Effective transcription of a small selection of these network members in CD34-DC was shown by their differential expression after sensitizing chemical exposure. In a first attempt to prove the functional relevance of VITOSENS® marker genes, the significantly differential protein expression of CCR2 and COX2 was established in sensitizer *versus* non-sensitizer-exposed CD34-DC. By proving the protein expression pattern of the marker genes to be equally discriminating compared to the gene expression responses, the relevance of the VITOSENS® discriminatory reaction for the biological process by which DC activate and present chemical haptens to naïve T cells is illustrated.

The KeratinoSens assay is a recently developed pathway-based assay that also fits the paradigm of toxicity testing in the twenty-first century. A reporter construct with luciferase activity was incorporated in a KC cell line, and upon exposure to skin sensitizers, the NF-E2-related factor 2 (Nrf2) transcription factor is activated and binds to antioxidant response element (ARE) in the promoter sequence of cytoprotective genes, thereby emitting quantifiable light [61]. The development of a pathway-based assay in a high-throughput setup may significantly enhance the rapid and efficient screening of chemicals for their possible skin-sensitizing properties.

Potency Assessment In Vitro

Improving the disease mechanism aids not only in the development of more precise prediction assays but also in a better extrapolation of the data for more accurate calculations of the risk for humans. As is the case for many diseases, skin sensitization is not an all-or-none phenomenon. There is evidence that dose–response relationships exist [124]. Appropriate dose–response data can provide important information on the potency of the material being tested, which may facilitate the development of more accurate risk assessments. Such data can now be generated from the mouse local lymph node assay (LLNA) for the sensitization phase, but it must be remembered that these animal data cannot be translated directly to human thresholds [125–127].

Earlier reports suggested that genes expressed in primary DC might be capable of classifying allergens into specific potency categories based on relative differences in gene expression [111]. Similar findings could be observed in the VITOSENS® assay. In this primary CD34-DC model, the gene expression dataset was extended by another *in vitro*-generated variable, namely, the IC_{20} or the concentration of the test chemical that yielded 20% cell damage. The relevance of including this IC_{20} as an explanatory variable was experimentally demonstrated by a clear

trend toward the chemical with lower dose (to induce a defined damage level) also being more sensitizing. By combining both the fold change in gene expression and IC_{20} in a robust multiple linear regression analysis, a potency value was modeled that closely fitted the LLNA potency data, and this is over the entire range from weak to extremely sensitizing chemicals (Fig. 2), with Spearman rank correlation coefficient = 0.91 and Pearson correlation coefficient = 0.79 [128].

This observation points to the feasibility of classifying sensitizers further into several potency classes based on *in vitro* data. Recently, multiple research groups have started to exploit this observation in their DC-based prediction assays. The above-mentioned LCSA, which is a coculture model composed of primary human KC and allogenic Mo-DC, considers the stimulation of CD86 expression and cell

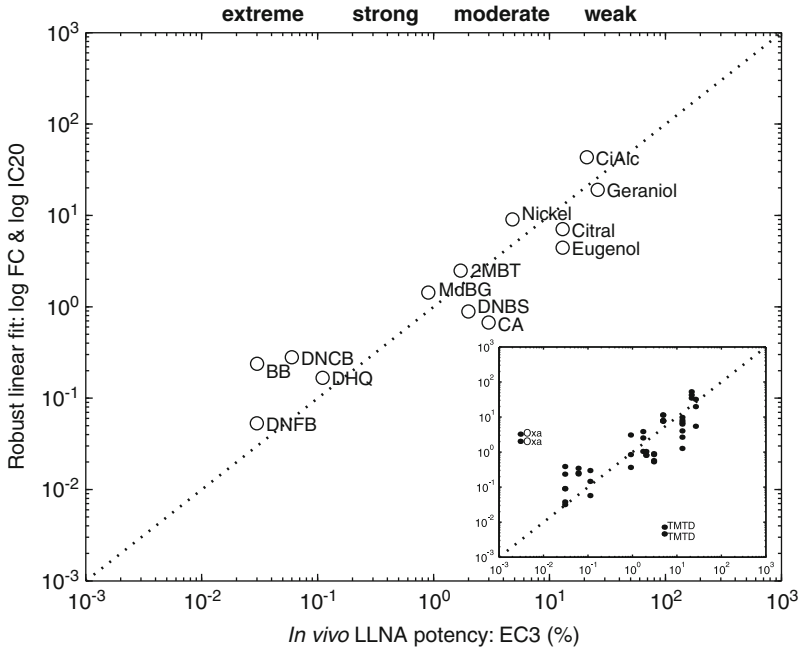


Fig. 2 Scatter plot of robust linear fit results from *in vitro* data (IC_{20} and fold changes) *versus in vivo* potency data. The *dashed line* is the identity diagonal, added as a visual guide. Per compound, the mean result of at least three replicate measurements is shown. After removal of the outlying compounds, Spearman rank correlation coefficient = 0.91, and Pearson correlation coefficient = 0.79. The four subcategories for *in vivo* potency classification are derived from (LLNA) classifications [129, 130] and indicated on top of the graph. The *inset* shows the individual results of each replicate measurement per compound. Data points which were considered as outliers are indicated by a cross and labeled by the exposure compound. Abbreviations: *FC* fold change, *LLNA* local lymph node assay, *EC3* 3-fold stimulation of lymph node proliferation in LLNA, *Oxa* oxazolone, *DNFB* dinitrofluorobenzene, *BB* Bandrowski's base, *DNCB* dinitrochlorobenzene, *DHQ* dihydroquinone, *MdBG* methyldibromoglutaronitrile, *2-MBT* 2-mercaptobenzothiazole, *DNBS* dinitrobenzenesulfonic acid, *CA* cinnamaldehyde, *Nickel* nickel sulfate, *TMTD* tetramethylthiuram disulfide, *Eug* eugenol, *CiAlc* cinnamic alcohol

viability of DC after exposure as a measure of skin-sensitizing potential of chemicals [30]. Also, in the DC-like cell line, THP-1 attempts to correlate *in vivo* potency data and *in vitro* predictions have been published [90, 131].

These findings may have major implications on human risk assessment. Potency information may contribute in the derivation of no- or minimal-effect levels, which is the threshold required for the induction of skin sensitization. Identification of maximum usage levels would be a clear step forward toward achieving increased chemical hygiene.

Lung Sensitization

A respiratory sensitizer is a substance that can induce a type 1 hypersensitivity reaction of the airways following inhalation of the substance. Hypersensitivity reactions induced by respiratory sensitizers may result in asthma, which is clinically characterized by wheezing, breathlessness, tightness in the chest, bronchoconstriction, and/or nasal congestion as most common symptoms. Asthma attributable to stimuli encountered at the workplace is defined as occupational asthma (OA), which is considered the most common occupational lung disease.

More than 350 agents have been implicated in the onset and development of respiratory allergy. A complete list of those agents can be found in research articles and reviews [132–138]. These agents are categorized into high molecular weight (HMW) and low molecular weight (LMW) agents.

The HMW compounds are generally of biological origin, e.g., proteins derived from animals, plants, foods, and enzymes, and act as complete allergens. Exposure to HMW agents is recognized by activation of antigen-presenting cells, leading to the production of specific IgE antibodies by B cells. Cross-linking of allergens with a specific IgE antibody on the surface of mast cells, basophils, and possibly macrophages, dendritic cells (DC), eosinophils, and platelets gives rise to a cascade of events that result in the influx and activation of inflammatory cells and in the release of preformed and newly formed inflammatory mediators that orchestrate the inflammatory process [27, 139, 140].

LMW compounds are generally chemically synthesized and represent an important subset of etiologic agents, including approximately 100 separate chemical entities. Although there are fewer LMW chemicals than HMW agents in the lists of occupational respiratory sensitizers, recent data indicate that LMW chemicals account for more new cases of OA caused by sensitization [17]. LMW chemical agents probably act as haptens becoming allergenic after conjugation with one or more body proteins [141]. Some of these induce asthma through a typical allergic Th2-type mechanism with attendant IgE production. The most well-known compounds in this group include acid anhydrides, metals, reactive dyes, and some pharmaceutical products. The majority of LMW compounds, however, do not consistently induce specific IgE antibodies, and the pathogenesis remains only partially elucidated [139, 141–144]. Specific IgE and IgG antibodies are found in

only a minority of cases and their precise role is unclear [145]. The airway inflammation process, however, is similar in IgE-dependent and IgE-independent asthma [146, 147] and is characterized by the presence of eosinophils, lymphocytes, mast cells, and thickening of the reticular basement membrane. Asthma due to diisocyanates, western red cedar, and amines are examples of IgE-independent causes of OA [148].

In Vitro Assays to Assess Respiratory Sensitization Hazard

As mentioned above, epithelial cells, DC, and macrophages are key cells involved in the development of respiratory sensitization.

The Epithelial Barrier

Bronchial and alveolar epithelial cell models have been extensively used to study the inflammatory potential of xenobiotics [149–161]. These models are good candidate assays to assess their use for respiratory sensitization screening. Recently, attempts to develop a transcriptomics-based *in vitro* assay based on alterations in gene expression of human epithelial cells after exposure to respiratory sensitizers and respiratory nonsensitizing chemicals have been initiated in order to identify genes that are able to discriminate between both groups of chemicals.

In the human bronchial epithelial BEAS-2B cell line, several genes have been identified that can distinguish three respiratory sensitizers from two irritants and one skin sensitizer [162]. Among the top-20 selective marker genes, *CASP9* is an interesting gene that can be associated with asthma and/or respiratory sensitization. When categorizing the 1,000 most discriminative genes into biological GO terms, 20 genes were associated with immune function. The majority of these genes have been shown to be related to airway inflammation, asthma, and respiratory sensitization (e.g., *CCL24*, *CEBPB*, *MIF*, and *TLR4*). Within this cellular model, the phosphatase and tensin homolog (PTEN) signaling pathway was identified as specific for respiratory sensitization.

In a similar study using the A549 alveolar epithelial cell model, a ranking of genes was obtained that reflected their potential to discriminate between respiratory (non-)sensitizing chemicals (e.g., *CLGN*, *CTLA4*, and *TXLNA*). Of these, *CTLA4* was associated with immune responses. When categorizing the top-1,000 genes into biological Gene Ontology (GO) terms, 22 genes were associated with immune function (e.g., *CCL4*, *CCL26*, *SPP1*, and *TLR2*). In this study, none of the canonical signaling pathways activated in alveolar epithelial cells were specific for respiratory sensitization [163].

The Dendritic Cell

Although airway DC are believed to be the conductor of immunological response, there is a growing awareness that these cells are activated by the presence of local immunomodulatory signals. A substantial proportion of these signals originate in the airway epithelium. DC also interact with epithelial cells by expressing adhesion molecules that not only anchor them strongly in the epithelium but also have a possible effect on their function [129, 130, 164–166].

Several DC-based assays (CD34-DC, Mo-DC, MUTZ-3, THP-1, KG-1, and U937), which have initially been developed for the purposes of skin sensitization testing [70], have been further exploited for respiratory allergen identification in the European Sens-it-iv program [97, 167, 168].

Macrophages

In the lung, resident alveolar macrophages are continuously encountering inhaled substances due to their exposed position in the alveolar lumen. Macrophages play a central role in the response to foreign materials and exhibit effects on alveolar and interstitial DC and T cells [169]. Besides *ex vivo* isolated alveolar macrophages, different human cell lines (THP-1, Mono-Mac-6) have been studied as a model of lung-derived macrophages [170–172].

Using THP-1-derived macrophages as *in vitro* model, different marker genes have been identified using gene expression studies that can distinguish respiratory sensitizers from respiratory nonsensitizers (e.g., *EIF4E*, *PDGFRB*, *SEMA7A*, and *ZFP36L2*). The function of the 1,000 most discriminative genes was linked to diverse biological processes, and 24 genes were associated with immune responses (e.g., *CCL20*, *IL10*, *TNF*, and *TLR4*). It could also be concluded that the canonical platelet-derived growth factor (PDGF) signaling pathway is probably the most specific one that was observed to be activated in macrophages in the context of respiratory sensitization, which is supported by existing literature information [173].

Coculture and 3D Models

A new area of research is the development of coculture models, and some of these have successfully been used for investigating both direct and indirect interactions between xenobiotics (e.g., cigarette smoke, ozone, and particles) and different cell types to mimic naturally occurring exposures. In the future, such culture systems may provide markers of immunoprocessing interactions.

Human monocytes and THP-1 macrophages were cocultured with monolayers of epithelial cells (human BEC, A549, renal adenocarcinoma epithelial cells) and the lung fibroblast strain HFL-1 [174]. A triple coculture model including macrophages and epithelial and dendritic cells which mimic the physiological communication

between these cell types has been developed [32] and might give new opportunities for sensitization screening of chemicals. Other examples of cocultures are BEAS-2B with human lung fibroblasts (HFL-1 or WISTAR-38) [175, 176], human umbilical vein endothelial cells (ECV304) [177], or eosinophils [153], and primary human BEC with alveolar macrophages [178].

In addition, 3D models of human airway tissues have been generated in order to study respiratory toxicity and infection. When cultivated *in vitro* on a polycarbonate filter at the air–liquid interface in a chemically defined medium, the human A549 alveolar cell resembles histologically the outer alveolar cell layers of the human lung alveoli (SkinEthic Laboratories, Nice, France). This model can be used for efficacy profiling of compounds or drugs for asthma treatment or type 1 allergy, or to better understand the safety potential of developed products applied by inhalation. Furthermore, Aufderheide *et al.* (2003) [179] reported on a technology allowing exposure of cells in a 3D culture to well-characterized aerosols (CULTEX®). MucilAir™, a novel *in vitro* cell model of the human airway epithelium, is a tool for acute and long-term toxicity testing of chemicals (Epithelix, Geneva, Switzerland). This *in vitro* system closely mimics the morphology and functions of the native human airway epithelium. There is little variation between batches that guarantees the reproducibility of the tests, and it has the ability to regenerate and repair after mechanical and chemical injuries.

Prospects

Chemical-induced allergy remains an important occupational health issue and a challenge to toxicologists. Although a considerable investment has been made in exploring opportunities to develop methods for hazard identification and characterization, there are, as yet, no validated alternative (*in silico*, *in chemico*, *in vitro*) methods available. Nevertheless, a number of emerging *in vitro* and *in silico* models show promise for use in the characterization of contact sensitization potential and should be further explored for their ability to identify and differentiate contact and respiratory sensitizers. Some of these systems may be useful in a weight-of-evidence approach or as a preliminary screen.

References

1. Behrendt H, Krämer U, Schäfer T, Kasche A, Eberlein-König B, Dasson U, Ring J (2001) Allergotoxicology—a research concept to study the role of environmental pollutants in allergy. Allerg Clin Immunol Int 13:122–128
2. Eggleston PA (2009) Complex interactions of pollutant and allergen exposures and their impact on people with asthma. Pediatrics 123(Suppl 3):S160–S167
3. EU (2006) Regulation (EC) No 1907/2006 of the European parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive

1999/45/EC and repealing Council Regulation (EEC) No 76/769/EEC and Commission Directive 91/155/EEC, 93/67/EEC, 93/105/EEC and 2000/21/EC. Official Journal of the European Union L396/1:1–849
4. EU (2003) Directive 2003/15/EC of the European Parliament and the Council of 27 February 2003 amending Council Directive 76/768/EEC on the approximation of the laws of the Member States relating to cosmetic products. Official Journal of the European Union L66: 26–35
5. NRC (2007) Toxicity testing in the 21st century: a vision and a strategy. The National Academies Press, Washington, DC
6. Maxwell G, Mackay C (2008) Application of a systems biology approach to skin allergy risk assessment. Altern Lab Anim 36:521–556
7. Basketter D, Maxwell G (2007) *In vitro* approaches to the identification and characterization of skin sensitizers. Cutan Ocul Toxicol 26:359–373
8. Kimber I, Agius R, Basketter DA, Corsini E, Cullinan P, Dearman RJ, Gimenez-Arnau E, Greenwell L, Hartung T, Kuper F, Maestrelli P, Roggen E, Rovida C (2007) Chemical respiratory allergy: opportunities for hazard identification and characterisation. The report and recommendations of ECVAM workshop 60. Altern Lab Anim 35:243–265
9. Vandebriel RJ, van Loveren H (2010) Non-animal sensitization testing: state-of-the-art. Crit Rev Toxicol 40:389–404
10. Graham C, Rosenkranz HS, Karol MH (1997) Structure-activity model of chemicals that cause human respiratory sensitization. Regul Toxicol Pharmacol 26:296–306
11. Fedorowicz A, Singh H, Soderholm S, Demchuk E (2005) Structure-activity models for contact sensitization. Chem Res Toxicol 18:954–969
12. Jarvis J, Seed MJ, Elton R, Sawyer L, Agius R (2005) Relationship between chemical structure and the occupational asthma hazard of low molecular weight organic compounds. Occup Environ Med 62:243–250
13. Seed MJ, Cullinan P, Agius RM (2008) Methods for the prediction of low-molecular-weight occupational respiratory sensitizers. Curr Opin Allergy Clin Immunol 8:103–109
14. Gerberick F, Aleksic M, Basketter D, Casati S, Karlberg AT, Kern P, Kimber I, Lepoittevin JP, Natsch A, Ovigne JM, Rovida C, Sakaguchi H, Schultz T (2008) Chemical reactivity measurement and the predictive identification of skin sensitisers. The report and recommendations of ECVAM Workshop 64. Altern Lab Anim 36:215–242
15. Aptula AO, Patlewicz G, Roberts DW (2005) Skin sensitization: reaction mechanistic applicability domains for structure-activity relationships. Chem Res Toxicol 18:1420–1426
16. Li Y, Tseng YJ, Pan D, Liu J, Kern PS, Gerberick GF, Hopfinger AJ (2007) 4D-fingerprint categorical QSAR models for skin sensitization based on the classification of local lymph node assay measures. Chem Res Toxicol 20:114–128
17. Maestrelli P, Boschetto P, Fabbri LM, Mapp CE (2009) Mechanisms of occupational asthma. J Allergy Clin Immunol 123:531–542
18. Mutschler J, Giménez-Arnau E, Foertsch L, Gerberick GF, Lepoittevin JP (2009) Mechanistic assessment of peptide reactivity assay to predict skin allergens with Kathon® CG isothiazolinones. Toxicol In Vitro 23:439–446
19. Gerberick GF, Vassallo JD, Foertsch LM, Price BB, Chaney JG, Lepoittevin JP (2007) Quantification of chemical peptide reactivity for screening contact allergens: a classification tree model approach. Toxicol Sci 97:417–427
20. Gerberick GF, Troutman JA, Foertsch LM, Vassallo JD, Quijano M, Dobson RL, Goebel C, Lepoittevin JP (2009) Investigation of peptide reactivity of pro-hapten skin sensitizers using a peroxidase-peroxide oxidation system. Toxicol Sci 112:164–174
21. Natsch A, Emter R, Ellis G (2009) Filling the concept with data: integrating data from different *in vitro* and *in silico* assays on skin sensitizers to explore the battery approach for animal-free skin sensitization testing. Toxicol Sci 107:106–121

22. Natsch A, Gfeller H (2008) LC-MS-based characterization of the peptide reactivity of chemicals to improve the *in vitro* prediction of the skin sensitization potential. Toxicol Sci 106:464–478
23. Roberts DW, Aptula AO, Patlewicz G, Pease C (2008) Chemical reactivity indices and mechanism-based read-across for non-animal based assessment of skin sensitisation potential. J Appl Toxicol 28:443–454
24. Lepoittevin JP (2006) Metabolism *versus* chemical transformation or pro-*versus* prehaptens? Contact Dermatitis 54:73–74
25. Holsapple MP, Afshari CA, Lehman-McKeeman LD (2009) Forum series: The "Vision" for toxicity testing in the 21st century: promises and conundrums. Toxicol Sci 107:307–308
26. Saint-Mezard P, Rosieres A, Krasteva M, Berard F, Dubois B, Kaiserlian D, Nicolas JF (2004) Allergic contact dermatitis. Eur J Dermatol 14:284–295
27. Verstraelen S, Bloemen K, Nelissen I, Witters H, Schoeters G, Van Den Heuvel R (2008) Cell types involved in allergic asthma and their use in *in vitro* models to assess respiratory sensitization. Toxicol In Vitro 22:1419–1431
28. Switalla S, Knebel J, Ritter D, Krug N, Braun A, Sewald K (2010) Effects of acute *in vitro* exposure of murine precision-cut lung slices to gaseous nitrogen dioxide and ozone in an air-liquid interface (ALI) culture. Toxicol Lett 196:117–124
29. Henjakovic M, Martin C, Hoymann HG, Sewald K, Ressmeyer AR, Dassow C, Pohlmann G, Krug N, Uhlig S, Braun A (2008) *Ex vivo* lung function measurements in precision-cut lung slices (PCLS) from chemical allergen-sensitized mice represent a suitable alternative to *in vivo* studies. Toxicol Sci 106:444–453
30. Wanner R, Sonnenburg A, Quatchadze M, Schreiner M, Peiser M, Zuberbier T, Stahlmann R (2010) Classification of sensitizing and irritative potential in a combined *in-vitro* assay. Toxicol Appl Pharmacol 245:211–218
31. BéruBé K, Aufderheide M, Breheny D, Clothier R, Combes R, Duffin R, Forbes B, Gaca M, Gray A, Hall I, Kelly M, Lethem M, Liebsch M, Merolla L, Morin JP, Seagrave J, Swartz MA, Tetley TD, Umachandran M (2009) *In vitro* models of inhalation toxicity and disease. The report of a FRAME workshop. Altern Lab Anim 37:89–141
32. Rothen-Rutishauser BM, Kiama SG, Gehr P (2005) A three-dimensional cellular model of the human respiratory tract to study the interaction with particles. Am J Respir Cell Mol Biol 32:281–289
33. Gibbs S, van de Sandt JJ, Merk HF, Lockley DJ, Pendlington RU, Pease CK (2007) Xenobiotic metabolism in human skin and 3D human skin reconstructs: a review. Curr Drug Metab 8:758–772
34. Grindon C, Combes R, Cronin MT, Roberts DW, Garrod JF (2007) An integrated decision-tree testing strategy for skin sensitisation with respect to the requirements of the EU REACH legislation. Altern Lab Anim 35:683–697
35. Ryan CA, Gerberick GF, Gildea LA, Hulette BC, Betts CJ, Cumberbatch M, Dearman RJ, Kimber I (2005) Interactions of contact allergens with dendritic cells: opportunities and challenges for the development of novel approaches to hazard assessment. Toxicol Sci 88: 4–11
36. Boverhof DR, Billington R, Gollapudi BB, Hotchkiss JA, Krieger SM, Poole A, Wiescinski CM, Woolhiser MR (2008) Respiratory sensitization and allergy: current research approaches and needs. Toxicol Appl Pharmacol 226:1–13
37. Smith Pease CK, Basketter DA, Patlewicz GY (2003) Contact allergy: the role of skin chemistry and metabolism. Clin Exp Dermatol 28:177–183
38. Bos JD, Meinardi MM (2000) The 500 Dalton rule for the skin penetration of chemical compounds and drugs. Exp Dermatol 9:165–169
39. Lepoittevin JP, Leblond I (1997) Hapten-peptide T cell receptor interactions: molecular basis for the recognition of haptens by T lymphocytes. Eur J Dermatol 7:151–154
40. Vocanson M, Hennino A, Rozieres A, Poyet G, Nicolas JF (2009) Effector and regulatory mechanisms in allergic contact dermatitis. Allergy 64:1699–1714

41. Naisbitt DJ (2004) Drug hypersensitivity reactions in skin: understanding mechanisms and the development of diagnostic and predictive tests. Toxicology 194:179–196
42. Bergeron C, Boulet LP (2006) Structural changes in airway diseases: characteristics, mechanisms, consequences, and pharmacologic modulation. Chest 129:1068–1087
43. Roychowdhury S, Svensson CK (2005) Mechanisms of drug-induced delayed-type hypersensitivity reactions in the skin. AAPS J 7:E834–E846
44. Hoath SB, Leahy DG (2003) The organization of human epidermis: functional epidermal units and phi proportionality. J Invest Dermatol 121:1440–1446
45. Toebak MJ, Gibbs S, Bruynzeel DP, Scheper RJ, Rustemeyer T (2009) Dendritic cells: biology of the skin. Contact Dermatitis 60:2–20
46. Steinman RM (2001) Dendritic cells and the control of immunity: enhancing the efficiency of antigen presentation. Mt Sinai J Med 68:160–166
47. Kimber I, Dearman RJ, Cumberbatch M, Huby RJ (1998) Langerhans cells and chemical allergy. Curr Opin Immunol 10:614–619
48. Kimber I, Basketter DA, Gerberick GF, Dearman RJ (2002) Review: allergic contact dermatitis. Int Immunopharmacol 2:201–211
49. Romani N, Holzmann S, Tripp CH, Koch F, Stoitzner P (2003) Langerhans cells—dendritic cells of the epidermis. APMIS 111:725–740
50. Ma J, Wang JH, Guo YJ, Sy MS, Bigby M (1994) *In vivo* treatment with anti-ICAM-1 and anti-LFA-1 antibodies inhibits contact sensitization-induced migration of epidermal Langerhans cells to regional lymph nodes. Cell Immunol 158:389–399
51. Price AA, Cumberbatch M, Kimber I, Ager A (1997) Alpha 6 integrins are required for Langerhans cell migration from the epidermis. J Exp Med 186:1725–1735
52. Sallusto F, Lanzavecchia A (2000) Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression. Immunol Rev 177:134–140
53. Enk AH, Katz SI (1992) Early molecular events in the induction phase of contact sensitivity. Proc Natl Acad Sci USA 89:1398–1402
54. Enk AH, Angeloni VL, Udey MC, Katz SI (1993) An essential role for Langerhans cell-derived IL-1β in the initiation of primary immune responses in skin. J Immunol 150: 3698–3704
55. Steinman R, Hoffman L, Pope M (1995) Maturation and migration of cutaneous dendritic cells. J Invest Dermatol 105:2S–7S
56. Jakob T, Udey MC (1998) Regulation of E-cadherin-mediated adhesion in Langerhans cell-like dendritic cells by inflammatory mediators that mobilize Langerhans cells *in vivo*. J Immunol 160:4067–4073
57. Theze J, Alzari PM, Bertoglio J (1996) Interleukin 2 and its receptors: recent advances and new immunological functions. Immunol Today 17:481–486
58. Sallusto F, Geginat J, Lanzavecchia A (2004) Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol 22:745–763
59. Cavani A, Sebastiani S, Nasorri F, Albanesi C, Girolomoni G (2002) Allergic contact dermatitis: effector and regulatory mechanisms. ACI Int 14:156–160
60. Corsini E, Mitjans M, Galbiati V, Lucchi L, Galli CL, Marinovich M (2009) Use of IL-18 production in a human keratinocyte cell line to discriminate contact sensitizers from irritants and low molecular weight respiratory allergens. Toxicol In Vitro 23:789–796
61. Emter R, Ellis G, Natsch A (2010) Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers *in vitro*. Toxicol Appl Pharmacol 245:281–290
62. Van Och FM, Van Loveren H, Van Wolfswinkel JC, Machielsen AJ, Vandebriel RJ (2005) Assessment of potency of allergenic activity of low molecular weight compounds based on IL-1α and IL-18 production by a murine and human keratinocyte cell line. Toxicology 210:95–109
63. Vandebriel RJ, Van Och F, van Loveren H (2005) *In vitro* assessment of sensitizing activity of low molecular weight compounds. Toxicol Appl Pharmacol 207:142–148
64. Basketter DA, Kimber I (2001) Predictive testing in contact allergy: facts and future. Allergy 56:937–943

65. Ryan CA, Hulette BC, Gerberick GF (2001) Approaches for the development of cell-based *in vitro* methods for contact sensitization. Toxicol In Vitro 15:43–55
66. Bonifas J, Hennen J, Dierolf D, Kalmes M, Blomeke B (2010) Evaluation of cytochrome P450 1 (CYP1) and *N*-acetyltransferase 1 (NAT1) activities in HaCaT cells: implications for the development of *in vitro* techniques for predictive testing of contact sensitizers. Toxicol In Vitro 24:973–980
67. Aiba S, Terunuma A, Manome H, Tagami H (1997) Dendritic cells differently respond to haptens and irritants by their production of cytokines and expression of co-stimulatory molecules. Eur J Immunol 27:3031–3038
68. Romani N, Gruner S, Brang D, Kampgen E, Lenz A, Trockenbacher B, Konwalinka G, Fritsch PO, Steinman RM, Schuler G (1994) Proliferating dendritic cell progenitors in human blood. J Exp Med 180:83–93
69. Caux C, Vanbervliet B, Massacrier C, Dezutter-Dambuyant C, de Saint-Vis B, Jacquet C, Yoneda K, Imamura S, Schmitt D, Banchereau J (1996) CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF + TNFα. J Exp Med 184:695–706
70. dos Santos GG, Reinders J, Ouwehand K, Rustemeyer T, Scheper RJ, Gibbs S (2009) Progress on the development of human *in vitro* dendritic cell based assays for assessment of the sensitizing potential of a compound. Toxicol Appl Pharmacol 236:372–382
71. Yoshida Y, Sakaguchi H, Ito Y, Okuda M, Suzuki H (2003) Evaluation of the skin sensitization potential of chemicals using expression of co-stimulatory molecules, CD54 and CD86, on the naive THP-1 cell line. Toxicol In Vitro 17:221–228
72. Ashikaga T, Yoshida Y, Hirota M, Yoneyama K, Itagaki H, Sakaguchi H, Miyazawa M, Ito Y, Suzuki H, Toyoda H (2006) Development of an *in vitro* skin sensitization test using human cell lines: the human Cell Line Activation Test (h-CLAT) I. Optimization of the h-CLAT protocol. Toxicol In Vitro 20:767–773
73. Sakaguchi H, Ashikaga T, Miyazawa M, Yoshida Y, Ito Y, Yoneyama K, Hirota M, Itagaki H, Toyoda H, Suzuki H (2006) Development of an *in vitro* skin sensitization test using human cell lines; human Cell Line Activation Test (h-CLAT) II. An inter-laboratory study of the h-CLAT. Toxicol In Vitro 20:774–784
74. Python F, Goebel C, Aeby P (2007) Assessment of the U937 cell line for the detection of contact allergens. Toxicol Appl Pharmacol 220:113–124
75. Azam P, Peiffer JL, Chamousset D, Tissier MH, Bonnet PA, Vian L, Fabre I, Ourlin JC (2006) The cytokine-dependent MUTZ-3 cell line as an *in vitro* model for the screening of contact sensitizers. Toxicol Appl Pharmacol 212:14–23
76. Becker D, Kuhn U, Lempertz U, Enk A, Saloga J, Knop J (1997) Flow-cytometric screening for the modulation of receptor-mediated endocytosis in human dendritic cells: implications for the development of an *in vitro* technique for predictive testing of contact sensitizers. J Immunol Methods 203:171–180
77. Tuschl H, Kovac R (2001) Langerhans cells and immature dendritic cells as model systems for screening of skin sensitizers. Toxicol In Vitro 15:327–331
78. Tuschl H, Kovac R, Weber E (2000) The expression of surface markers on dendritic cells as indicators for the sensitizing potential of chemicals. Toxicol In Vitro 14:541–549
79. Degwert J, Steckel F, Hoppe U, Kligman LH (1997) *In vitro* model for contact sensitization: I. Stimulatory capacities of human blood-derived dendritic cells and their phenotypical alterations in the presence of contact sensitizers. Toxicol In Vitro 11:613–618
80. Aiba S, Manome H, Yoshino Y, Tagami H (2000) *In vitro* treatment of human transforming growth factor-β1-treated monocyte-derived dendritic cells with haptens can induce the phenotypic and functional changes similar to epidermal Langerhans cells in the initiation phase of allergic contact sensitivity reaction. Immunology 101:68–75
81. Coutant KD, de Fraissinette AB, Cordier A, Ulrich P (1999) Modulation of the activity of human monocyte-derived dendritic cells by chemical haptens, a metal allergen, and a staphylococcal superantigen. Toxicol Sci 52:189–198

82. Hulette BC, Ryan CA, Gerberick GF (2002) Elucidating changes in surface marker expression of dendritic cells following chemical allergen treatment. Toxicol Appl Pharmacol 182:226–233
83. Hulette BC, Ryan CA, Gildea LA, Gerberick GF (2005) Relationship of CD86 surface marker expression and cytotoxicity on dendritic cells exposed to chemical allergen. Toxicol Appl Pharmacol 209:159–166
84. Staquet MJ, Sportouch M, Jacquet C, Schmitt D, Guesnet J, Peguet-Navarro J (2004) Moderate skin sensitizers can induce phenotypic changes on *in vitro* generated dendritic cells. Toxicol In Vitro 18:493–500
85. Aeby P, Wyss C, Beck H, Griem P, Scheffler H, Goebel C (2004) Characterization of the sensitizing potential of chemicals by *in vitro* analysis of dendritic cell activation and skin penetration. J Invest Dermatol 122:1154–1164
86. Rougier N, Redziniak G, Mougin D, Schmitt D, Vincent C (2000) *In vitro* evaluation of the sensitization potential of weak contact allergens using langerhans-like dendritic cells and autologous T cells. Toxicology 145:73–82
87. De Smedt AC, Van Den Heuvel RL, Zwi Berneman N, Schoeters GE (2001) Modulation of phenotype, cytokine production and stimulatory function of CD34 + -derived DC by $NiCl_2$ and SDS. Toxicol In Vitro 15:319–325
88. De Smedt AC, Van Den Heuvel RL, Van Tendeloo VF, Berneman ZN, Schoeters GE (2005) Capacity of CD34+ progenitor-derived dendritic cells to distinguish between sensitizers and irritants. Toxicol Lett 156:377–389
89. Boisleve F, Kerdine-Romer S, Rougier-Larzat N, Pallardy M (2004) Nickel and DNCB induce CCR7 expression on human dendritic cells through different signalling pathways: role of TNF-α and MAPK. J Invest Dermatol 123:494–502
90. Sakaguchi H, Ashikaga T, Miyazawa M, Kosaka N, Ito Y, Yoneyama K, Sono S, Itagaki H, Toyoda H, Suzuki H (2009) The relationship between CD86/CD54 expression and THP-1 cell viability in an *in vitro* skin sensitization test—human Cell Line Activation Test (h-CLAT). Cell Biol Toxicol 25:109–126
91. Sakaguchi H, Ryan C, Ovigne JM, Schroeder KR, Ashikaga T (2010) Predicting skin sensitization potential and inter-laboratory reproducibility of a human Cell Line Activation Test (h-CLAT) in the European Cosmetics Association (COLIPA) ring trials. Toxicol In Vitro 24:1810–1820
92. Pichowski JS, Cumberbatch M, Dearman RJ, Basketter DA, Kimber I (2000) Investigation of induced changes in interleukin 1β mRNA expression by cultured human dendritic cells as an *in vitro* approach to skin sensitization testing. Toxicol In Vitro 14:351–360
93. Pichowski JS, Cumberbatch M, Dearman RJ, Basketter DA, Kimber I (2001) Allergen-induced changes in interleukin 1 beta (IL-1β) mRNA expression by human blood-derived dendritic cells: inter-individual differences and relevance for sensitization testing. J Appl Toxicol 21:115–121
94. Aiba S, Manome H, Nakagawa S, Mollah ZU, Mizuashi M, Ohtani T, Yoshino Y, Tagami H (2003) p38 Mitogen-activated protein kinase and extracellular signal-regulated kinases play distinct roles in the activation of dendritic cells by two representative haptens, $NiCl_2$ and 2,4-dinitrochlorobenzene. J Invest Dermatol 120:390–399
95. Jugde F, Boissier C, Rougier-Larzat N, Corlu A, Chesne C, Semana G, Heresbach D (2005) Regulation by allergens of chemokine receptor expression on *in vitro*-generated dendritic cells. Toxicology 212:227–238
96. Toebak MJ, Pohlmann PR, Sampat-Sardjoepersad SC, von Blomberg BM, Bruynzeel DP, Scheper RJ, Rustemeyer T, Gibbs S (2006) CXCL8 secretion by dendritic cells predicts contact allergens from irritants. Toxicol In Vitro 20:117–124
97. Toebak MJ, Moed H, von Blomberg MB, Bruynzeel DP, Gibbs S, Scheper RJ, Rustemeyer T (2006) Intrinsic characteristics of contact and respiratory allergens influence production of polarizing cytokines by dendritic cells. Contact Dermatitis 55:238–245

98. De Smedt AC, Van Den Heuvel RL, Van Tendeloo VF, Berneman ZN, Schoeters GE, Weber E, Tuschl H (2002) Phenotypic alterations and IL-1β production in CD34(+) progenitor- and monocyte-derived dendritic cells after exposure to allergens: a comparative analysis. Arch Dermatol Res 294:109–116
99. Antonios D, Ade N, Kerdine-Romer S, Assaf-Vandecasteele H, Larange A, Azouri H, Pallardy M (2009) Metallic haptens induce differential phenotype of human dendritic cells through activation of mitogen-activated protein kinase and NF-kappaB pathways. Toxicol In Vitro 23:227–234
100. Rustemeyer T, Preuss M, von Blomberg BM, Das PK, Scheper RJ (2003) Comparison of two *in vitro* dendritic cell maturation models for screening contact sensitizers using a panel of methacrylates. Exp Dermatol 12:682–691
101. Verheyen GR, Schoeters E, Nuijten JM, Van Den Heuvel RL, Nelissen I, Witters H, Van Tendeloo VF, Berneman ZN, Schoeters GE (2005) Cytokine transcript profiling in CD34 + -progenitor derived dendritic cells exposed to contact allergens and irritants. Toxicol Lett 155:187–194
102. Casati S, Aeby P, Basketter DA, Cavani A, Gennari A, Gerberick GF, Griem P, Hartung T, Kimber I, Lepoittevin JP, Meade BJ, Pallardy M, Rousset F, Ribinstenn G, Sallusto F, Verheyen GR, Zuang V (2005) Dendritic cells as a tool for the predictive identification of skin sensitisation hazard. Altern Lab Anim 33:47–62
103. Szameit S, Vierlinger K, Farmer L, Tuschl H, Noehammer C (2008) Microarray-based *in vitro* test system for the discrimination of contact allergens and irritants: identification of potential marker genes. Clin Chem 54:525–533
104. Cluzel-Tailhardat M, Bonnet-Duquennoy M, de Queral DP, Vocanson M, Kurfurst R, Courtellemont P, Le Varlet B, Nicolas JF (2007) Chemicals with weak skin sensitizing properties can be identified using low-density microarrays on immature dendritic cells. Toxicol Lett 174:98–109
105. Schoeters E, Verheyen GR, Van Den Heuvel R, Nelissen I, Witters H, Van Tendeloo VF, Schoeters GE, Berneman ZN (2005) Expression analysis of immune-related genes in CD34 (+) progenitor-derived dendritic cells after exposure to the chemical contact allergen DNCB. Toxicol In Vitro 19:909–913
106. Schoeters E, Nuijten JM, Van Den Heuvel RL, Nelissen I, Witters H, Schoeters GE, Van Tendeloo VF, Berneman ZN, Verheyen GR (2006) Gene expression signatures in CD34 + -progenitor-derived dendritic cells exposed to the chemical contact allergen nickel sulfate. Toxicol Appl Pharmacol 216:131–149
107. Ryan CA, Gildea LA, Hulette BC, Dearman RJ, Kimber I, Gerberick GF (2004) Gene expression changes in peripheral blood-derived dendritic cells following exposure to a contact allergen. Toxicol Lett 150:301–316
108. Szameit S, Vierlinger K, Farmer L, Tuschl H, Noehammer C (2009) Gene expression studies in cultured dendritic cells: new indicators for the discrimination of skin sensitizers and irritants *in vitro*. Clin Exp Allergy 39:856–868
109. Schoeters E, Verheyen GR, Nelissen I, Van Rompay AR, Hooyberghs J, Van Den Heuvel RL, Witters H, Schoeters GE, Van Tendeloo VF, Berneman ZN (2007) Microarray analyses in dendritic cells reveal potential biomarkers for chemical-induced skin sensitization. Mol Immunol 44:3222–3233
110. Gildea LA, Ryan CA, Foertsch LM, Kennedy JM, Dearman RJ, Kimber I, Gerberick GF (2006) Identification of gene expression changes induced by chemical allergens in dendritic cells: opportunities for skin sensitization testing. J Invest Dermatol 126:1813–1822
111. Hooyberghs J, Schoeters E, Lambrechts N, Nelissen I, Witters H, Schoeters G, Van Den Heuvel R (2008) A cell-based *in vitro* alternative to identify skin sensitizers by gene expression. Toxicol Appl Pharmacol 231:103–111
112. Lambrechts N, Verstraelen S, Lodewyckx H, Felicio A, Hooyberghs J, Witters H, Van Tendeloo V, Van Cauwenberge P, Nelissen I, Van Den Heuvel R et al (2009) THP-1

monocytes but not macrophages as a potential alternative for CD34+ dendritic cells to identify chemical skin sensitizers. Toxicol Appl Pharmacol 236:221–230
113. Guironnet G, Dalbiez-Gauthier C, Rousset F, Schmitt D, Peguet-Navarro J (2000) *In vitro* human T cell sensitization to haptens by monocyte-derived dendritic cells. Toxicol In Vitro 14:517–522
114. Moser JM, Sassano ER, del Leistritz C, Eatrides JM, Phogat S, Koff W, Drake DR 3rd (2009) Optimization of a dendritic cell-based assay for the *in vitro* priming of naive human CD4+ T cells. J Immunol Methods 353:8–19
115. Regnier M, Patwardhan A, Scheynius A, Schmidt R (1998) Reconstructed human epidermis composed of keratinocytes, melanocytes and Langerhans cells. Med Biol Eng Comput 36: 821–824
116. Facy V, Flouret V, Regnier M, Schmidt R (2005) Reactivity of Langerhans cells in human reconstructed epidermis to known allergens and UV radiation. Toxicol In Vitro 19:787–795
117. Schreiner M, Peiser M, Briechle D, Stahlmann R, Zuberbier T, Wanner R (2007) A loose-fit coculture of activated keratinocytes and dendritic cell-related cells for prediction of sensitizing potential. Allergy 62:1419–1428
118. Schreiner M, Peiser M, Briechle D, Stahlmann R, Zuberbier T, Wanner R (2008) A new dendritic cell type suitable as sentinel of contact allergens. Toxicology 249:146–152
119. Spielmann H, Hoffmann S, Liebsch M, Botham PA, Fentem JH, Eskes C, Roquet R, Cotovio J, Cole T, Worth A, Heylings J, Jones P, Robles C, Kandárová H, Gamer A, Remmele M, Curren R, Raabe H, Cockshott A, Gerner I, Zuang V (2007) The ECVAM international validation study on *in vitro* tests for acute skin irritation: report on the validity of the EPISKIN and EpiDerm assays and on the Skin Integrity Function Test. Altern Lab Anim 35:559–604
120. Poumay Y, Coquette A (2007) Modelling the human epidermis *in vitro*: tools for basic and applied research. Arch Dermatol Res 298:361–369
121. Coquette A, Berna N, Vandenbosch A, Rosdy M, De Wever B, Poumay Y (2003) Analysis of interleukin-1α (IL-1α) and interleukin-8 (IL-8) expression and release in *in vitro* reconstructed human epidermis for the prediction of *in vivo* skin irritation and/or sensitization. Toxicol In Vitro 17:311–321
122. Ryan CA, Kimber I, Basketter DA, Pallardy M, Gildea LA, Gerberick GF (2007) Dendritic cells and skin sensitization: biological roles and uses in hazard identification. Toxicol Appl Pharmacol 221:384–394
123. Lambrechts N, Vanheel H, Hooyberghs J, De Boever P, Witters H, Van Den Heuvel R, Van Tendeloo V, Nelissen I, Schoeters G (2010) Gene markers in dendritic cells unravel pieces of the skin sensitization puzzle. Toxicol Lett 196:95–103
124. van Loveren H, Cockshott A, Gebel T, Gundert-Remy U, de Jong WH, Matheson J, McGarry H, Musset L, Selgrade MK, Vickers C (2008) Skin sensitization in chemical risk assessment: report of a WHO/IPCS international workshop focusing on dose-response assessment. Regul Toxicol Pharmacol 50:155–199
125. ECHA (2008) Guidance on information requirements and chemical safety assessment Chapter R.8: dose [concentration]-response regarding human health. Online available at http://guidance.echa.europa.eu/docs/guidance_document/information_requirements_r8_en.pdf?vers=20_08_08
126. Kimber I, Basketter DA, Dearman RJ (2009) Chemical allergens—what are the issues? Toxicology 268:139–142
127. Jowsey IR, Basketter DA, Westmoreland C, Kimber I (2006) A future approach to measuring relative skin sensitising potency: a proposal. J Appl Toxicol 26:341–350
128. Lambrechts N, Vanheel H, Nelissen I, Witters H, Van Den Heuvel R, Van Tendeloo V, Schoeters G, Hooyberghs J (2010) Assessment of chemical skin sensitizing potency by an *in vitro* assay based on human dendritic cells. Toxicol Sci 196:95–103
129. Hammad H, Lambrecht BN (2008) Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. Nat Rev Immunol 8:193–204

130. Proud D (2008) The pulmonary epithelium in health and disease. Wiley, Chichester
131. Mitjans M, Galbiati V, Lucchi L, Viviani B, Marinovich M, Galli CL, Corsini E (2010) Use of IL-8 release and p38 MAPK activation in THP-1 cells to identify allergens and to assess their potency *in vitro*. Toxicol In Vitro 24:1803–1809
132. Chan-Yeung M, Lam S (1986) Occupational asthma. Am Rev Respir Dis 133:686–703
133. Bernstein DI (1997) Allergic reactions to workplace allergens. J Am Med Assoc 278: 1907–1913
134. Cullinan P, Newman Taylor AJ (1997) Aetiology of occupational asthma. Clin Exp Allergy 27:41–46
135. Chan-Yeung M, Obata H, Dittrick M, Chan H, Abboud R (1999) Airway inflammation, exhaled nitric oxide, and severity of asthma in patients with western red cedar asthma. Am J Respir Crit Care Med 159:1434–1438
136. van Kampen V, Merget R, Baur X (2000) Occupational airway sensitizers: an overview on the respective literature. Am J Ind Med 38:164–218
137. Mapp CE (2001) Agents, old and new, causing occupational asthma. Occup Environ Med 58:354–360
138. Bernstein DI (2003) Occupational asthma caused by exposure to low-molecular-weight chemicals. Immunol Allergy Clin North Am 23:221–234
139. Mapp CE, Boschetto P, Maestrelli P, Fabbri LM (2005) Occupational asthma. Am J Respir Crit Care Med 172:280–305
140. Bloemen K, Verstraelen S, Van Den Heuvel R, Witters H, Nelissen I, Schoeters G (2007) The allergic cascade: review of the most important molecules in the asthmatic lung. Immunol Lett 113:6–18
141. Sastre J, Vandenplas O, Park HS (2003) Pathogenesis of occupational asthma. Eur Respir J 22:364–373
142. Wisnewski AV, Liu Q, Miller JJ, Magoski N, Redlich CA (2002) Effects of hexamethylene diisocyanate exposure on human airway epithelial cells: *in vitro* cellular and molecular studies. Environ Health Perspect 110:901–907
143. Mapp CE, Fryer AA, Marzo ND, Pozzato V, Padoan M, Boschetto P, Strange RC, Hemmingsen A, Spiteri MA (2002) Glutathione *S*-transferase GSTP1 is a susceptibility gene for occupational asthma induced by isocyanates. J Allergy Clin Immunol 109:867–872
144. Chan-Yeung M, Malo JL, Tarlo SM, Bernstein L, Gautrin D, Mapp C, Newman-Taylor A, Swanson MC, Perrault G, Jaques L, Blanc PD, Vandenplas O, Cartier A, Becklake MR (2003) Proceedings of the first Jack Pepys Occupational Asthma Symposium. Am J Respir Crit Care Med 167:450–471
145. Malo JL, Chan-Yeung M (2001) Occupational asthma. J Allergy Clin Immunol 108:317–328
146. Bentley AM, Maestrelli P, Saetta M, Fabbri LM, Robinson DS, Bradley BL, Jeffery PK, Durham SR, Kay AB (1992) Activated T-lymphocytes and eosinophils in the bronchial mucosa in isocyanate-induced asthma. J Allergy Clin Immunol 89:821–829
147. Turato G, Saetta M (2000) Why does airway obstruction persist in asthma due to low-molecular-weight agents? A pathologistis view. Occup Med 15:445–454
148. Chan-Yeung M, Malo JL (1994) Aetiological agents in occupational asthma. Eur Respir J 7:346–371
149. Stellato C, Brummet ME, Plitt JR, Shahabuddin S, Baroody FM, Liu MC, Ponath PD, Beck LA (2001) Expression of the C-C chemokine receptor CCR3 in human airway epithelial cells. J Immunol 166:1457–1461
150. Pichavant M, Charbonnier AS, Taront S, Brichet A, Wallaert B, Pestel J, Tonnel AB, Gosset P (2005) Asthmatic bronchial epithelium activated by the proteolytic allergen Der p1 increases selective dendritic cell recruitment. J Allergy Clin Immunol 115:771–778
151. Erlemann KR, Cossette C, Gravel S, Lesimple A, Lee GJ, Saha G, Rokach J, Powell WS (2007) Airway epithelial cells synthesize the lipid mediator 5-oxo-ETE in response to oxidative stress. Free Radic Biol Med 42:654–664
152. van Wetering S, Zuyderduyn S, Ninaber DK, van Sterkenburg MA, Rabe KF, Hiemstra PS (2007) Epithelial differentiation is a determinant in the production of eotaxin-2 and -3 by bronchial epithelial cells in response to IL-4 and IL-13. Mol Immunol 44:803–811

153. Wong CK, Wang CB, Ip WK, Tian YP, Lam CW (2005) Role of p38 MAPK and NF-kB for chemokine release in coculture of human eosinophils and bronchial epithelial cells. Clin Exp Immunol 139:90–100
154. Rippmann JF, Schnapp A, Weith A, Hobbie S (2005) Gene silencing with STAT6 specific siRNAs blocks eotaxin release in IL-4/TNFα stimulated human epithelial cells. FEBS Lett 579:173–178
155. Matsukura S, Kokubu F, Kuga H, Kawaguchi M, Ieki K, Odaka M, Suzuki S, Watanabe S, Takeuchi H, Adachi M (2003) Differential regulation of eotaxin expression by IFN-γ in airway epithelial cells. J Allergy Clin Immunol 111:1337–1344
156. Pascal LE, Tessier DM (2004) Cytotoxicity of chromium and manganese to lung epithelial cells *in vitro*. Toxicol Lett 147:143–151
157. Lee YM, Kim HA, Park HS, Lee SK, Nahm DH (2003) Exposure to toluene diisocyanate (TDI) induces IL-8 production from bronchial epithelial cells: effect of pro-inflammatory cytokines. J Korean Med Sci 18:809–812
158. Krakauer T (2000) Pentoxifylline inhibits ICAM-1 expression and chemokine production induced by proinflammatory cytokines in human pulmonary epithelial cells. Immunopharmacology 46:253–261
159. Kwong KYC, Literat A, Zhu NL, Huang HH, Li C, Jones CA, Minoo P (2004) Expression of transforming growth factor beta (TGF-β1) in human epithelial alveolar cells: a pro-inflammatory mediator independent pathway. Life Sci 74:2941–2957
160. Moriyuki K, Nagataki M, Sekiguchi F, Nishikawa H, Kawabata A (2008) Signal transduction for formation/release of interleukin-8 caused by a PAR2-activating peptide in human lung epithelial cells. Regul Pept 145:42–48
161. Richter M, Cantin AM, Beaulieu C, Cloutier A, Larivee P (2003) Zinc chelators inhibit eotaxin, RANTES, and MCP-1 production in stimulated human airway epithelium and fibroblasts. Am J Physiol Lung Cell Mol Physiol 285:L719–L729
162. Verstraelen S, Nelissen I, Hooyberghs J, Witters H, Schoeters G, Van Cauwenberge P, Van Den Heuvel R (2009) Gene profiles of a human bronchial epithelial cell line after *in vitro* exposure to respiratory (non-)sensitizing chemicals: identification of discriminating genetic markers and pathway analysis. Toxicology 255:154–159
163. Verstraelen S, Nelissen I, Hooyberghs J, Witters H, Schoeters G, Van Cauwenberge P, Van Den Heuvel R (2009) Gene profiles of a human alveolar epithelial cell line after *in vitro* exposure to respiratory (non-)sensitizing chemicals: identification of discriminating genetic markers and pathway analysis. Toxicol Lett 185:16–22
164. Willart MA, Lambrecht BN (2009) The danger within: endogenous danger signals, atopy and asthma. Clin Exp Allergy 39:12–19
165. van Tongeren J, Reinartz SM, Fokkens WJ, de Jong EC, van Drunen CM (2008) Interactions between epithelial cells and dendritic cells in airway immune responses: lessons from allergic airway disease. Allergy 63:1124–1135
166. Roggen EL, Lindstedt M, Borrebaeck C, Verheyen GR (2006) Interactions between dendritic cells and epithelial cells in allergic disease. Toxicol Lett 162:71–82
167. Rovida C, Basketter D, Casati S, de Silva O, Hermans H, Kimber I, Manou I, Weltzien HU, Roggen E (2007) Management of an integrated project (Sens-it-iv) to develop *in vitro* tests to assess sensitisation. Altern Lab Anim 35:317–322
168. Roggen E, Aufderheide M, Cetin Y, Dearman RJ, Gibbs S, Hermanns I, Kimber I, Rovida C, Warhait DB, Uhlig S, Casati S (2008) The development of novel approaches to the identification of chemical and protein respiratory allergens. Altern Lab Anim 36:591–598
169. Lambrecht BN (2006) Alveolar macrophage in the driver's seat. Immunity 24:366–368
170. Verstraelen S, Wens B, Hooyberghs J, Nelissen I, Witters H, Schoeters G, Cauwenberge PV, Heuvel RV (2008) Gene expression profiling of *in vitro* cultured macrophages after exposure to the respiratory sensitizer hexamethylene diisocyanate. Toxicol In Vitro 22:1107–1114
171. Mitjans M, Viviani B, Lucchi L, Galli CL, Marinovich M, Corsini E (2008) Role of p38 MAPK in the selective release of IL-8 induced by chemical allergen in naive THP-1 cells. Toxicol In Vitro 22:386–395

172. Elms J, Beckett PN, Griffin P, Curran AD (2001) Mechanisms of isocyanate sensitisation. An *in vitro* approach. Toxicol In Vitro 15:631–634
173. Verstraelen S, Nelissen I, Hooyberghs J, Witters H, Schoeters G, Van Cauwenberge P, Van Den Heuvel R (2009) Gene profiles of THP-1 macrophages after *in vitro* exposure to respiratory (non-)sensitizing chemicals: identification of discriminating genetic markers and pathway analysis. Toxicol In Vitro 23:1151–1162
174. Striz I, Slavcev A, Kalanin J, Jaresova M, Rennard SI (2001) Cell-cell contacts with epithelial cells modulate the phenotype of human macrophages. Inflammation 25:241–246
175. Lang DS, Jorres RA, Mucke M, Siegfried W, Magnussen H (1998) Interactions between human bronchoepithelial cells and lung fibroblasts after ozone exposure *in vitro*. Toxicol Lett 96–97:13–24
176. Lang DS, Schocker H, Hockertz S (2001) Effects of crocidolite asbestos on human bronchoepithelial-dependent fibroblast stimulation in coculture: the role of IL-6 and GM-CSF. Toxicology 159:81–98
177. Mogel M, Kruger E, Krug HF, Seidel A (1998) A new coculture-system of bronchial epithelial and endothelial cells as a model for studying ozone effects on airway tissue. Toxicol Lett 96–97:25–32
178. Ishii H, Hayashi S, Hogg JC, Fujii T, Goto Y, Sakamoto N, Mukae H, Vincent R, van Eeden SF (2005) Alveolar macrophage-epithelial cell interaction following exposure to atmospheric particles induces the release of mediators involved in monocyte mobilization and recruitment. Respir Res 6:87
179. Aufderheide M, Knebel JW, Ritter D (2003) An improved *in vitro* model for testing the pulmonary toxicity of complex mixtures such as cigarette smoke. Exp Toxicol Pathol 55:51–57

Male Reprotoxicity and Endocrine Disruption

Sarah Campion, Natasha Catlin, Nicholas Heger, Elizabeth V. McDonnell, Sara E. Pacheco, Camelia Saffarini, Moses A. Sandrof, and Kim Boekelheide

Abstract Mammalian reproductive tract development is a tightly regulated process that can be disrupted following exposure to drugs, toxicants, endocrine-disrupting chemicals (EDCs), or other compounds via alterations to gene and protein expression or epigenetic regulation. Indeed, the impacts of developmental exposure to certain toxicants may not be fully realized until puberty or adulthood when the reproductive tract becomes sexually mature and altered functionality is manifested. Exposures that occur later in life, once development is complete, can also disrupt the intricate hormonal and paracrine interactions responsible for adult functions, such as spermatogenesis. In this chapter, the biology and toxicology of the male reproductive tract is explored, proceeding through the various life stages including *in utero* development, puberty, adulthood, and senescence. Special attention is given to the discussion of EDCs, chemical mixtures, low-dose effects, transgenerational effects, and potential exposure-related causes of male reproductive tract cancers.

Keywords Androgen insensitivity syndrome · Bisphenol A · Chemical mixtures · Cryptorchidism · Diethylstilbestrol · Endocrine-disrupting chemicals · Hypospadias · Low-dose effects · Phthalates · Testicular dysgenesis syndrome · Testicular germ cell tumors · Transgenerational effects · Vinclozolin

Sarah Campion, Natasha Catlin, Nicholas Heger, Elizabeth V. McDonnell, Sara E. Pacheco, Camelia Saffarini, Moses A. Sandrof and Kim Boekelheide (✉)
Department of Pathology and Laboratory Medicine, Brown University, Providence, RI 02912, USA
e-mail: kim_boekelheide@brown.edu

A. Luch (ed.), *Molecular, Clinical and Environmental Toxicology*,
Experientia Supplementum 101, DOI 10.1007/978-3-7643-8340-4_11,

Introduction

The concept of developmental origins of disease is certainly not new, yet only recently has the idea been supported by epidemiological research. In the mid-1990s, Dr. David Barker observed a correlation between low birth weight and an increased incidence of diseases later in life such as stroke, hypertension, coronary artery disease, and diabetes [1, 2]. Barker suggested that human fetuses permanently adapt their developing cellular physiology and metabolism to survive in conditions of inadequate maternal nutrition in preparation for a presumptively similar nutrient-poor postnatal environment. The result is an enhanced susceptibility to certain diseases in postnatal life due to an altered cellular metabolism that cannot adjust to conditions of adequate nutrition. One such example of this is the famine of the Dutch Hunger Winter from late 1944 to early 1945, during which thousands of people in the Netherlands experienced severely reduced caloric intake due to a German embargo on food imports and an early winter freeze that hindered canal transport. During this time, thousands of pregnant women were exposed to famine conditions. The children of this cohort have shown higher incidences of hyperlipidemia [3], altered DNA methylation patterns [4], early onset coronary artery disease [5], altered glucose tolerance and insulin secretion [6], and obesity [7] compared to unexposed controls. These findings lend credence to Barker's hypotheses and demonstrate that patterns set during critical programming windows of development can adversely influence health later in life.

While epidemiological studies have underscored the significance of the developmental origins of adult disease, human health risk assessment for environmental toxicant exposure is grounded primarily in animal studies. Many specific study designs are aimed at characterizing the potential for effects on reproduction and fertility and are performed in conjunction with developmental toxicity assessments. Although each regulatory agency has different testing requirements (reviewed in [8]), multigenerational tests represent the cornerstone of developmental and reproductive toxicity testing. This type of testing ensures detection of effects caused by pre- and postnatal exposures, effects with delayed onset, and effects that are transgenerational and could be missed with single generation testing [9]. A well-designed multigenerational test should examine three dose levels of the chemical in question that together produce a gradation of toxic effects; some reprotoxicity should be seen with the highest dose, while the lowest dose should exhibit minimal to no effects. This information can then be used to establish a "no observed adverse effect level" (NOAEL) that helps extrapolate data to determine safe exposure levels for humans.

Rats are the most commonly used species in multigenerational reproduction studies. Generally, healthy parental (F0) animals (both male and female) between 5 and 9 weeks of age are administered the chemical orally (through diet, drinking water, or gavage) on a daily basis for 10 weeks prior to and during mating; exposure in the 10 weeks prior to mating allows for the manifestation of effects on gametogenesis. Administration continues daily during gestation and through the weaning of the F1 offspring. The chemical continues to be administered to selected F1

generation male and female offspring into adulthood and through mating and production of an F2 generation until the F2 offspring are weaned. Using this study design, both F1 and F2 offspring are continuously exposed *in utero* from conception until birth and during the preweaning period; in this way, effects from exposures throughout development can be detected, including those that occur in the peripubertal and young adult phases [10].

There are several different reproductive endpoints that can be evaluated as part of a multigenerational study. While all animals are monitored for body weight and mating and fertility indices, there are endpoints specifically relevant to the male animal, including gross examination and morphology of the reproductive organs, observation of developmental effects, and measurement of sperm effects. Weight and histopathological analyses of the testes, epididymides, and accessory sex glands, including the prostate and seminal vesicles, are conducted; because these accessory sex glands are androgen dependent, they may reflect changes in the animal's endocrine status or testicular function. Normal physical development may also be effected by exposure, including testicular descent, anogenital distance, and structure of the external genitalia. Finally, evaluations of sperm number, morphology, and motility can suggest exposure-related damage; sperm number data are derived from counts of homogenization-resistant spermatid heads in the testis and epididymis [11]. Histopathological evaluation can be a sensitive indicator of damage; some signs of adverse effects include changes in seminiferous tubule diameter, spermatid head retention, vacuolization, sloughing, and germ cell apoptosis, among other endpoints.

While this study design is ideal for identifying potential adverse outcomes of a specific chemical or group of chemicals, it does not look at the mechanism of action for the chemicals in question. The National Research Council of the National Academies [12] has recently published a report that seeks to incorporate testing strategies that will move toward understanding the mode of action of chemicals. In "Toxicity in the 21st Century: A Vision and a Strategy," they outline a systems biology approach driven by high-throughput testing strategies that utilize modern tools and toxicogenomic testing and that will provide important mechanistic data [13]. While finding *in vitro*/*ex vivo* cell models that mimic the complex interactions of reproductive and developmental cell types presents a potential challenge to the high-throughput testing strategies envisioned in the report, there are some promising models in development that are able to assess perturbations of the developing fetus and reproductive organs. As described by Chapin and Steadman [14], stem cells are able to differentiate into different cell types and therefore could play an important role in developing new assays to predict developmental and reproductive toxicity *in vitro*. Furthermore, stem cells can be evaluated for toxic responses in their undifferentiated state or during the process of differentiation. In addition to the development of *in vitro* models for reprotoxicity testing, future safety assessments will likely incorporate epigenetic evaluations as the relationship between epigenetic changes and health outcomes is better understood, particularly as more information becomes available on the potential transgenerational effects of toxicants [13].

We now look at the biology and toxicology of the male reproductive tract, starting with early development, followed by puberty, adulthood, and senescence.

Biology and Toxicology of the Developing Male Reproductive Tract

The Early Fetal Gonad

Prior to the onset of sexual differentiation, the human gonad is composed of proliferating coelomic and mesenchymal cells, primordial germ cells, and endothelial cells (gestational weeks 7–8) [15]. This early fetal gonad has the precursors of both male (Wolffian) and female (Mullerian) reproductive tracts and maintains the ability to differentiate into either sex [16]. Development of a male reproductive system requires two crucial events: formation of the testis, and differentiation and maintenance of the Wolffian duct through adulthood [17]. This process is induced by Mullerian-inhibiting substance (MIS, also known as anti-Mullerian hormone, AMH), which is produced by the pre-Sertoli cells of the early testis. In the presence of MIS and testosterone, the Mullerian duct regresses and the Wolffian duct differentiates into the vas deferens and epididymis [18]; in their absence, the Wolffian duct regresses and the Mullerian duct develops into the oviduct, uterus, and upper vagina, resulting in a female phenotype [19].

Genetic Regulation of Male Sexual Differentiation

Sexual differentiation is primarily controlled by the SRY sex-determining gene on the short arm of the Y chromosome. When present, development of the male pathway is initiated through activation of molecular and cellular cascades that lead to testis formation and regression of the Mullerian duct [17, 20, 21]. The SRY gene likely acts as a genetic switch, encoding for transcription factors that allow cells of the early gonad to further develop; organizational changes of the cells are seen around 36 h after the initial expression of SRY [22]. Sertoli cells are the first to differentiate within the gonad; there is strong evidence that it is the pre-Sertoli cell that expresses SRY [23], initiating differentiation of gonad to testis [24]. Changes in the early testis include seminiferous cord formation through the organization of pre-Sertoli cells around germ cells, increase in size of the XY gonads compared to the XX gonads, and development of a characteristic blood vessel on the periphery of the XY gonad [18]. Formation of the Sertoli cell lineage is strictly controlled by the expression of SRY-activated genes [23].

There are three major genes activated by SRY that are critical to male sexual differentiation—SOX9 (SRY-box containing gene 9), Fgf9 (fibroblast growth factor-9), and Sf1 (steroidogenic factor 1) [25, 26]. Transcription in the promoter region of SRY binding sites is activated when WT1 encodes a transcription factor that binds to the SRY protein [27]. First, null mutations in both WT1 and Sf1 can alter the development of the gonad and may be involved in initiating signals that

control expansion of the genital ridge [28, 29]. Sf1 may also activate the transcription of hormones that masculinize both Sertoli and Leydig cells [21]. Second, because the transcription factor SOX9 can induce full male differentiation in the absence of SRY, it likely plays an important role in Sertoli cell formation [21]. At the time of sex determination, SOX9 moves into the nucleus and binds to the promoter site on the gene that codes for anti-Mullerian hormone, providing a pathway to male phenotype development [21]. Finally, the Fgf9 gene is responsible for the proliferation and differentiation of Sertoli cell precursors and aids in testis cord formation by initiating the migration of mesonephric cells into the gonad [21]. Following activation of these genetic regulators of sex differentiation, early cell types become apparent in the developing gonad.

Precursors of supportive reproductive cell types of both sexes, the Sertoli cell of the testis and the granulosa cell of the ovary, are believed to be present in the early gonad [18]. It is also likely that in the early gonad there is a single precursor of steroidogenic cells of both the male (Leydig cells) and female (theca cells) [30]. Unlike Sertoli cells, which require the expression of SRY for development, fetal Leydig cells do not express SRY or SOX9, indicating that they differentiate as a result of the paracrine action of Sertoli cells [17], which is driven by two main signaling molecules: desert hedgehog (DHH) and platelet-derived growth factors (PDGFs) [31]. Sertoli cells, without further involvement of SRY, direct the development of other cell types in the early testis, including peritubular myoid cells, endothelial cells, and uncharacterized cells within the interstitial space [18, 23]. These additional cell types aid in seminiferous cord formation by surrounding Sertoli cells with peritubular myoid cells and help form the blood–testis barrier (BTB) by creating a basal lamina that, in conjunction with Sertoli cells, encloses germ cells within the testis cords [18].

Hormonal Regulation of Male Sexual Differentiation

Hormonal control of male sexual differentiation begins with the formation of the Sertoli cells. Sertoli cells secrete anti-Mullerian hormone, inducing the regression of the Mullerian duct and allowing Leydig cells to initiate further differentiation of the Wolffian duct and accessory glands through secretion of androgens and insulin-like factor 3 (Insl3). Production of these hormones is partially regulated by the presence of human chorionic gonadotropin (hCG) and luteinizing hormone (LH) [17, 32]. The Wolffian duct is further stabilized through secretion of testosterone by the Leydig cell, allowing for differentiation of the vas deferens, epididymis, seminal vesicles, and ejaculatory duct from the Wolffian duct; once developed, these become dependent on androgens secreted by Leydig cells [17]. Differentiation of the urethra, prostate gland, penis and scrotum, and tissue between the anus and genital orifice is dependent on the conversion of testosterone to dihydrotestosterone (DHT) by 5α-reductase [16, 33]. Insl3 helps induce testicular descent toward the end of sexual development [16, 17].

Testicular descent from the intra-abdominal site to an extracorporeal site (scrotum) allows the mature testis to maintain normal spermatogenesis later in life [32].

The cranial suspensory ridge (CSL) anchors the upper poles of the regressing mesonephrous and developing testis [34]. The genitoinguinal ligament (gubernaculum) connects the lower poles of the testis and the epididymis with the future inguinal canal [15]. Testicular descent occurs in two major phases: first, hormonal regulation of complex anatomical rearrangements begins around gestational week 25 in humans, stimulating transabdominal descent of the testes [15]. This phase is primarily regulated by Insl3, which initiates thickening of the gubernaculum by binding to its receptor, LGR8 (leucine-rich repeat-containing G protein coupled receptor 8) [34, 35]. Thickening of the gubernaculum anchors the testis in the inguinal region until the second phase of testicular descent, the inguinoscrotal phase, which begins approximately 10 weeks after the completion of transabdominal descent [15]. While androgen expression is not necessary for transabdominal descent, it is required in the second phase to induce regression of the CSL [36]. When these two steps are complete, the testes are pulled into the scrotum and further anchored during gestational weeks 35–40, completing the process of testicular descent [15, 32].

Congenital Abnormalities

Both genetic (SRY-Sox9) and hormonal (androgens) regulations are necessary in mammals for the development and organization of the male reproductive tract. If this delicate process is disturbed, either by genetic abnormalities or chemical exposure, reproductive tract disorders can result, including cryptorchidism, hypospadias, impaired spermatogenesis, and testicular cancer [37]. The increased incidence of these abnormalities has led to a theory of testicular dysgenesis syndrome (TDS), which is discussed in further detail later in this chapter [37]. Cryptorchidism and hypospadias are fairly frequent congenital abnormalities that have been studied in detail. They may occur as isolated disorders or may be associated with other congenital syndromes, such as those described below.

Cryptorchidism is the failure of one or both testes to descend properly into the scrotal sac. The most common congenital birth defect in male children, it occurs at a rate of 2–4% in full-term births; about 50% of cryptorchid testes spontaneously descend in the first few months after birth [38]. The etiology is largely unknown; however, it is likely that both genetic and environmental factors (acting as endocrine disruptors) contribute to cryptorchid outcomes [39]. Several genetic causes and polymorphisms associated with cryptorchidism have been identified in human patients, including mutations in Insl3 and its receptor, RXFP2, as well as in the androgen receptor (AR) gene [which is associated with androgen insensitivity syndrome (AIS)] [38]. While there is a strong association of cryptorchidism with infertility and testicular cancer, the mechanisms underlying this association are unknown [38].

Hypospadias results from abnormal penile and urethral development, commonly arising from a defect in the midline fusion of the male urethra, leading to a misplaced urethral meatus. It is the most frequent genital malformation in male newborns [40]. As with cryptorchidism, the increased incidence of hypospadias has

largely been attributed to environmental factors [37]. Despite this, there are several genes involved in penile development that may influence a hypospadic outcome, including HOXA13, FGF10, and those involved with androgen synthesis and action (LH receptor gene, 17-hydroxysteroid reductase, and AR) [40].

In addition, several other congenital disorders have been studied with an eye to the etiology of disease states: congenital adrenal hyperplasia (CAH), AIS, Klinefelter syndrome, and hypothalamic hypogonadism may also result from chromosomal or genetic abnormalities or may be induced by chemical exposure during fetal development.

CAH refers to a group of autosomal recessive disorders caused by a reduced or complete lack of activity of one of the steroidogenic enzymes involved in cortisol biosynthesis in the adrenal cortex [41]. The specific enzymatic defect results in different biochemical and clinical phenotypes whose severity depends on the location of normal enzymatic action in the adrenal steroidogenic pathway and the degree of enzyme deficiency. In about 90–95% of cases, CAH is caused by a deficiency in the 21-hydroxylase enzyme, resulting in decreased levels of aldosterone or cortisol. Lacking the negative feedback normally present, the pituitary secretes hormones that stimulate glucocorticoid and/or mineralcorticoid production. Increased glucocorticoid and mineralcorticoid precursors accumulate and are shunted into the androgen synthesis pathway, leading to hyperandrogenism. This excessive androgen production influences the development of external genitalia in both sexes [42]. Hyperandrogenic effects that present later in life include precocious pseudopuberty (in both sexes) and infertility resulting from excess androgen and estrogen production [41].

AIS is characterized by a resistance to androgens and can manifest in a variety of ways depending on the degree of AR disruption. Generally, males with AIS present with a female phenotype due to one or various AR mutations. In the extreme case of complete AIS, a lack of activity at the androgen receptor prevents testicular descent and further development of the external male genitalia; however, normal production of MIS during fetal development inhibits uterus and fallopian tube development, resulting in an XY male genotype with a female phenotype [43].

Hypothalamic hypogonadism, also known as Kallmann syndrome, is caused by improper migration of gonadotropin-releasing hormone (GnRH)-secreting neurons to the hypothalamus during fetal development. Because GnRH stimulates follicle-stimulating hormone (FSH) and LH hormone production in the pituitary, which stimulates the gonads, a GnRH deficiency leads to decreased or absent function of the testes. Symptoms can include the absence of secondary sexual characteristics, delayed puberty, underdeveloped testes, and/or microphallus. Several genetic mutations known to cause Kallmann syndrome are reviewed by Fechner *et al*. [44].

Klinefelter syndrome refers to a group of chromosomal disorders characterized by one or more additional X chromosomes added to a normal male karyotype. The classic form of Klinefelter is 47,XXY; this occurs at a rate of 1:500 and represents the most common chromosomal disorder among men [45, 46]. Because the X chromosome carries genes responsible for normal testis function, brain growth and development, and many others, X chromosome polysomy carries significant consequences [45, 47]. The most common signs of Klinefelter syndrome are

spermatogenic and steroidogenic dysfunctions, and as a result, the disorder often remains undiagnosed until later in life. Other symptoms include small, firm testes with slightly decreased testicular volume, low serum testosterone resulting in erectile dysfunction and poor libido, and primary testicular failure resulting in infertility (azoospermia or severe oligospermia) [45].

Permeability of Placental Barrier

It is well established that *in utero* exposure to cigarette smoke, drugs, and alcohol can adversely affect fetal development. However, it was originally believed that the fetus was uniquely protected from exposure to chemicals due to the nature of the placental barrier. Charged with the tasks of delivering nutrients, removing wastes, modulating endocrine activity, and regulating immune surveillance, the placental barrier is imperfect by design and fails to keep all harmful compounds in the maternal circulation from reaching the fetus. Moreover, the developing fetus does not have the protective factors of an adult—it has immature detoxification, metabolic, excretory, and immune functions that increase its susceptibility to toxic insult. The idea that the placenta formed a perfect barrier around the fetus was challenged in the 1950s and 1960s when chemical-induced birth defects demonstrated the fragility of the unborn fetus to insults by maternal exposures. In the 1950s, *in utero* thalidomide exposure led to drug-induced phocomelia (limb shortening); by 1961, this sedative drug was withdrawn from the market. Similarly, consumption of methylmercury-contaminated shellfish by pregnant mothers has led to neurological dysfunction in their children, most prominently in Minimata Bay, Japan, where a population was exposed from 1932 to 1968. The symptoms that together are known as "Minimata disease" were first recognized in that area in 1956.

Examples of insults from environmental exposure are not limited to the human population. The 1962 publication of Rachel Carson's seminal work, "Silent Spring," suggested that the overuse of the pesticide dichlorodiphenyltrichloroethane (DDT) affected nontarget species such as birds, impacting their ability to reproduce and resulting in a decline in wildlife populations. Legislation over the past 50 years has been aimed at minimizing environmental pollution and mandating stronger regulations for drug safety. The legacy of these pivotal events has been instrumental in shifting public attitudes toward an increased awareness of the environment, chemical exposures, and the vulnerability of the developing fetus.

Endocrine-Disrupting Chemicals

The ability of chemicals to affect reproduction and development has garnered significant attention. The term "endocrine-disrupting chemical" (EDC) was coined in 1993, when it was suggested that certain chemicals could mimic endogenous hormones,

effectively disrupting hormone homeostasis. Further, it was suggested that exposure to these chemicals was responsible for the increased incidences of lowered fertility, intersexed animals, and disrupted sexual development observed in certain wildlife species [48]. Implicated compounds included a wide variety of chemical pesticides (DDT, methoxychlor, tributyltin, and others), polychlorinated biphenyls (PCBs), dioxin (i.e., TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin), and many other types of anthropogenic chemicals. It is clear that toxicant-induced modulation of the endocrine system can have myriad downstream effects on reproduction and development. Side effects from exposure are not necessarily negative; however, from a pharmacological perspective, modulation of hormonal signaling could have potentially therapeutic effects. Receptor antagonists such as tamoxifen and flutamide are routinely used in the treatment of breast and prostate cancer, respectively. Other drugs, such as ethinyl estradiol (EE), a stable derivative of estradiol used in birth control pills, alter hormonal signaling through estrogen receptor (ER) binding and inhibit the release of gonadotropins, effectively preventing ovulation. Birth control pills are the most widely used form of contraception in the United States [49]. Not surprisingly, the widespread use of oral contraceptives has resulted in the detection of EE in treated waste water and has been linked to endocrine disruptive effects in fish, such as altered hormone levels, feminized or intersex species, and increased production of the estrogen-dependent protein vitellogenin [50, 51].

Epidemiological Analysis of EDCs

Despite this evidence, the vast majority of chemicals in our environment have yet to be adequately assessed for their potential as endocrine disruptors. The 1996 passage of the US Food Quality Protection Act calls on the US Environmental Protection Agency (US-EPA) to develop and implement an Endocrine Disruptor Screening Program (EDSP) to test chemicals for their ability to affect the endocrine systems of humans and other wildlife. Similarly, the 2007 legislation of the European Union's REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) will eventually require manufacturers to identify hazards associated with their products based on the toxicity of the individual constituents. Although testing has not yet commenced, laying the framework for appropriate testing methodology is an important and necessary first step in determining the endocrine-disrupting potential of compounds in our environment.

Primary research has been able to demonstrate the endocrine-disrupting activities of some of these chemicals. However, it has been difficult to establish a definitive link between exposure and altered reproductive development and function. In animal experiments of EDC exposure, the incidence of endocrine disruption is often widely varied due to differences in dose, timing, metabolism, and animal model. Additionally, the mechanisms driving these effects are not fully understood, further complicating the extrapolation of primary data to clinical relevance for human health and reproduction. A case of drug-induced endocrine disruption in 1971 underscores some of the challenges faced in conducting epidemiological

analyses. In that year, physicians at Massachusetts General Hospital observed eight cases of vaginal clear cell adenocarcinoma in young women between the ages of 14 and 22. Because it is a rare cancer normally seen in older women, these cases drew attention, and another link was found: 7 of the 8 girls were born to women prescribed the synthetic estrogen diethylstilbestrol (DES) during the first trimester of pregnancy [52]. DES was initially prescribed to pregnant women as an off-label use to help prevent miscarriage and support a healthy pregnancy. However, a full analysis later suggested an array of reproductive tract problems in both sons and daughters exposed to DES during the first trimester of development, prompting the conditions of pregnancy to be considered a contraindication for use [53–55].

"DES daughters" are known to be at an increased risk of cancer, ectopic pregnancy, preterm delivery, infertility, and alterations to uterine and cervical structure [56]. Research has suggested that some of these effects may be mediated through multiple mechanisms, including the activation of proto-oncogenes, delayed expression of genes regulating reproductive structure, and altered gene methylation patterns [57–59]. "DES sons" have shown reproductive problems as well, including epididymal cysts, hypospadias, cryptorchidism, and microphallus [56]. Experiments in male mice and rats demonstrate that this estrogen analog causes Leydig cell dysfunction via an ERα-dependent mechanism, along with a reduction in insulin-like hormone 3 (Insl3), resulting in improper gubernacular development and testis maldescent [60–62]. DES also causes decreased testosterone production resulting from decreased synthesis of steroidogenic proteins cytochrome 17α-hydroxylase/17,20-lyase/17,20-desmolase (Cyp17a1) and steroidogenic acute regulatory protein (Star) [63]. A major challenge in identifying putative mechanisms is the highly variable incidence of the assorted reproductive effects observed in DES sons and daughters. This is likely due to differences in exposure duration, timing, dosing, and genetic heterogeneity among a diverse human population. While current findings may not fully explain all manifestations of endocrine disruption following developmental DES exposure, the spectrum of reproductive defects observed demonstrates human fetal susceptibility to endocrine active compounds.

Testicular Dysgenesis Syndrome

In the years since EDCs first took the spotlight, interest has expanded beyond simple estrogen mimicry. It is now known that compounds can alter endocrine activity in varying ways, covering the spectrum of pro- and antiestrogenic, androgenic, and goitrogenic effects. At the same time, epidemiologic research has noted adverse trends in reproductive health and fertility of men in developed countries. In particular, epidemiologists have cited an increase in the incidence of reproductive congenital malformations and adult-onset diseases over the past 70 years [64]. Congenital defects include varying degrees of cryptorchidism and hypospadias, as well as other associated anomalies of the epididymis, seminal vesicles, and vas deferens. Adult-onset diseases of concern include alterations in sperm quantity, morphology, and motility, as

well as testis germ cell cancer. Observation of these symptoms has resulted in the proposal of a "testicular dysgenesis syndrome" (TDS), suggesting that these effects may share a common etiology in altered reproductive development [37]. According to this hypothesis, perturbations to the developmentally critical *in utero* or perinatal environment, possibly due to EDC exposure, may result in subsequent dysgenesis of the male reproductive tract. Epidemiological support for this theory comes from examination of the contralateral testis of adult men with testis germ cell cancer, where focal areas of altered seminiferous tubule morphology, Leydig cell hyperplasia, and immature Sertoli cells have been observed. This suggests that altered germ cells resulting from a disrupted developmental environment may play a role in the development of cancer. [65]. While the prevalence of the diseases and disorders that comprise TDS is highly variable between individuals, the manifestation of one may be considered a risk factor for developing another, as with unresolved cryptorchidism and subsequent testicular cancer.

While some rare genetic defects and point mutations can lead to altered reproductive structure and function, the overall prevalence of these genetic abnormalities cannot explain the increasing incidence or variable expressivity of the full array of TDS disorders. As a result, researchers have looked toward the use of transgenic animal models to examine the potential molecular mechanisms of the various TDS phenotypes observed in humans. Previous work in developmental biology has observed TDS-like effects with the loss or disruption of genes regulating reproductive growth and development. Testes from Fgf9-null mouse pups at embryonic day 18.5 exhibit a wide range of altered testicular phenotypes with disorganization of seminiferous cords and disrupted peritubular myoid cell organization [66]. Dax-1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region on chromosome X, gene 1) deficient males exhibit infertility due to obstruction of the efferent ducts and rete testis as well as Leydig and Sertoli cell tumors [67]. Loss of the gene Insl3 causes bilateral intra-abdominal cryptorchidism with secondary infertility and testicular atrophy [68]. The aforementioned models have provided valuable information about the multifaceted roles of signaling molecules and transcription factors in male reproductive development and function. While each model produces one or two of the phenotypes described in human TDS cases, other undesirable pathological effects such as lung hypoplasia and ovarian-like testis organization limit their usefulness as a model for human TDS.

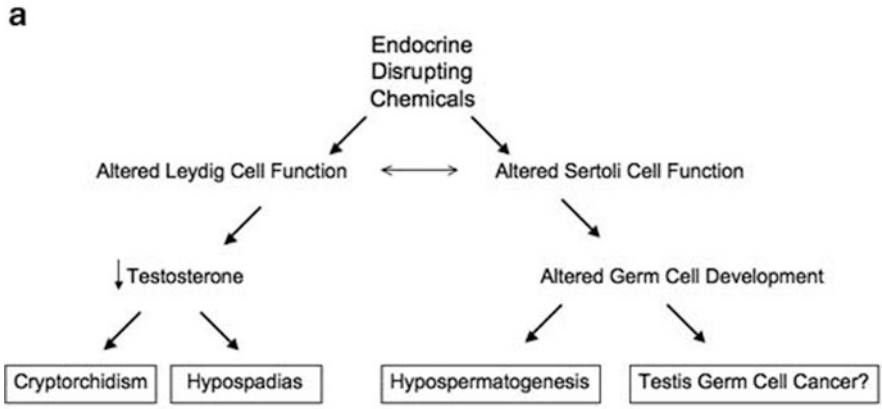
Phthalates

The phthalate ester class of chemicals has recently received considerable attention due to its widespread use and reported endocrine disruption activities. Phthalate esters are high production volume chemicals used as plasticizers in polyvinyl chloride (PVC) plastics to impart flexibility. They are also used as emulsifying agents, surfactants, and lubricants in numerous industrial, medical, and cosmetic products. As these compounds are not covalently bound to the PVC polymer, they can leach with age, use, and ultraviolet light exposure, making them available for

biological exposure [69]. Detectable levels of various phthalate metabolites have been observed in the urine of the general population by the United States Centers for Disease Control and Prevention [70]. Additionally, critically ill neonates receiving intensive medical treatment can be exposed to up to 10–20 mg/kg of phthalate esters due to the use of PVC-based medical devices [71]. As such, phthalates have been associated with the development of TDS due to the potential for significant exposure during development and the induction of reproductive tract defects in rats following gestational exposure (Fig. 1) [72–74]. Importantly, the suppression of fetal steroidogenesis has resulted in an expression of "serious concern" for phthalate exposure of intensively medically treated infants by an expert panel for the Center of the Evaluation of Risks to Human Reproduction [75].

Early work on the endocrine-disrupting activities of phthalates by the National Toxicology Program (NTP) in 1991 demonstrated alterations to reproductive tract structure, seminiferous tubule degeneration, and lowered sperm counts in male pups exposed to di-(*n*-butyl) phthalate (DBP) during mid to late gestation [76]. Interestingly, these effects were not observed in the parental generation, suggesting that additional studies would be needed to more fully characterize the effects of developmental phthalate exposure. While estrogenic properties of phthalates had previously been reported, subsequent research pointed toward an antiandrogenic mechanism, as female rats gestationally exposed to DBP exhibited no change in reproductive organ weights, estrous cyclicity, or vaginal opening, as was observed with weakly estrogenic compounds [77–79]. Experiments in rats showed that DBP and other similarly acting phthalates exhibit dose-dependent effects on the developing male reproductive tract, including cryptorchidism, hypospadias, hypospermatogenesis, increased seminiferous cord diameter, decreased anogenital distance, and the formation of multinucleated germ cells (MNGs) [79–82]. The antiandrogenic mode of action was confirmed when it was shown that DBP and its active monoester metabolite, mono-(*n*-butyl) phthalate (MBP), could lower both the expression of steroidogenic genes and intratesticular testosterone content without interacting with the AR [83–85]. It is now accepted that decreased Insl3 and lowered testosterone production underlie the manifestation of cryptorchidism, and hypospadias and altered secondary reproductive structures, respectively [86]. Although the pathogenesis of MNG formation is still unknown, it is believed that these rogue cells may arise from altered Sertoli-germ cell communication and altered cytokinesis.

The effects of *in utero* exposure to phthalates have been well characterized, demonstrating clear antiandrogenic effects on the rat male reproductive tract. While most work has focused on the rat as a susceptible species to the antiandrogenic effects of phthalates, published work in 2007 attempted to extend the rat model to mice in order to explore potential species differences in response. The findings of this study showed that *in utero* exposure of mice to equivalent doses of endocrine active phthalates exhibited similar toxicokinetics and caused the same induction of MNG formation and alterations to seminiferous cords seen in the rat model. Interestingly, however, these fetal murine effects were observed in the absence of any measureable differences in steroidogenic gene expression or intratesticular testosterone content. This important species difference in response suggested that



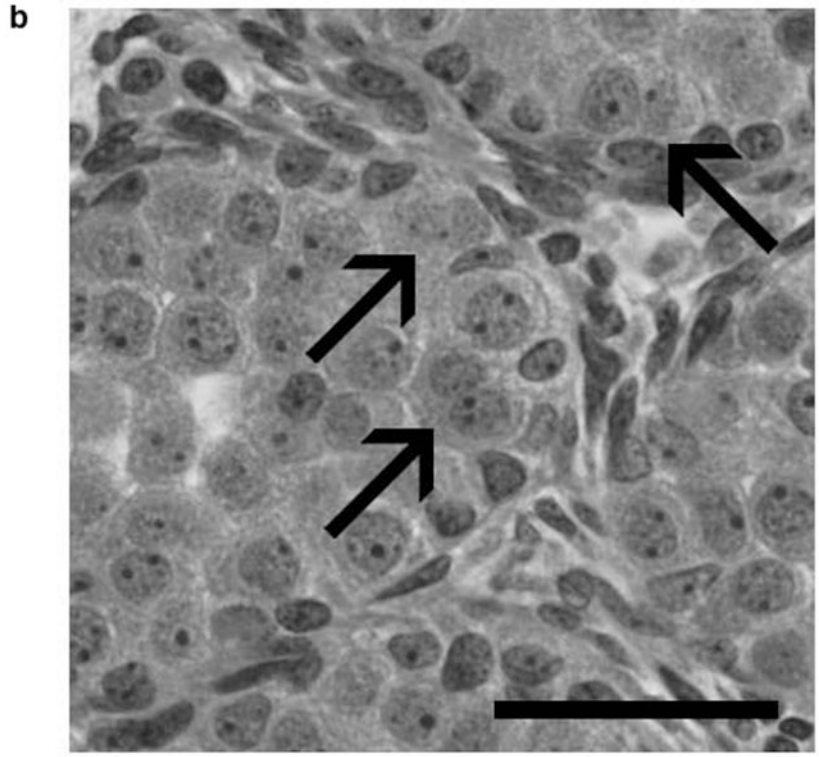


Fig. 1 Endocrine-disrupting chemical effects upon the fetal testis. (**a**) Endocrine-disrupting chemicals alter both Leydig cell and Sertoli cell function, producing downstream abnormalities. (**b**) As an example, multinucleated germ cells (MNGs, *arrows*) are induced in gd18 fetal Fischer rat testis following 2 days of maternal exposure to 250 mg/kg/day di-(*n*-butyl) phthalate (DBP). H&E staining. Bar = 50 μm

the effects of developmental phthalate exposure on seminiferous cords (increased diameter, MNG induction) are not mechanistically linked to lowered steroidogenesis and testosterone production.

The divergent response observed with developmental phthalate exposure in mice and rats highlights the inherent difficulty in relying on single species toxicity testing approaches. Differences in susceptibility and sensitivity hold particular importance for the extrapolation of risks to humans following toxicant exposure. As *in utero* experimentation is both costly and labor intensive, *in vitro* organ culture has been explored as a straightforward and inexpensive method for studying cellular effects of phthalates. Unfortunately, these models have been fraught with many limitations, including inconsistent responses in testosterone production following phthalate exposure, a treatment-related loss of germ cells, and failure of MNG induction [87–90]. For these reasons, *in vitro* approaches have thus far appeared inadequate for examining at least some phthalate-induced effects on the testis. However, the single species *in utero* exposure models used with phthalate research to date do not allow for determining whether the disparity in effect on Leydig cell steroidogenesis is an intrinsic response of the species, or an inherent property of the testis itself. This could be examined using a cross-species xenotransplant model, consisting of mouse and rat testis transplants into species either resistant (mouse) or sensitive (rat) to phthalate-induced effects on steroidogenesis. The interspecies approach would allow for direct assessment of the testicular response to phthalates on both a gene and histological level, while examining the role of host-specific factors in species responses.

Biology and Toxicology of the Pubertal Male Reproductive Tract

In humans, a neonatal surge of testosterone during the first 4 months of life leads to testosterone levels that resemble those of a healthy adult male [16]. By 6 months of age, testosterone levels decline to near negligible levels and remain low until puberty, when the male acquires adult reproductive capabilities, develops secondary sex characteristics, and experiences a growth spurt [16].

The hypothalamus–pituitary–gonadal (HPG) axis is critical to sexual maturation, both at the fetal and pubertal stages. To initiate puberty, the hypothalamus secretes gonadotropin releasing hormone (GnRH), which causes an increase in nocturnal pulsatile gonadotropin (LH and FSH) secretion from the pituitary gland [91]. In adult males, pulsatile secretion occurs approximately every 90 min; the frequency with which this occurs is an important factor in normal gonadal response [92]. When the gonadotropin pulse frequency (and thus secretion of gonadal sex hormones) reaches a critical level, secondary sexual characteristics begin to form; this marks the beginning of phenotypic puberty [91]. While FSH is not used until sperm maturation, LH is released during sleep alongside pulsatile GnRH, causing gonadal stimulation and inducing Leydig cell hyperplasia that leads to testosterone release [93]. During puberty, the testis cords hollow to form seminiferous tubules, and the germ cells migrate toward the periphery of the tubules where they begin to

differentiate into sperm [21]. The HPG axis is critical for both gonadal development and steroid production; if it is disrupted, a hypogonadal state can result, leading to abnormalities such as the congenital disorders previously described.

It is during puberty that the blood–testis barrier develops as an important barrier to both environmental toxicants and the immune system—this occurs alongside the proliferation of spermatogonia to primary spermatocytes [94]. Adjacent Sertoli cells in the seminiferous epithelium form tight junctions (TJ) using actin-based adherens junctions (AJ) characterized by actin filament bundles. The bundles join the Sertoli cell plasma membrane and the subsurface cistern of the endoplasmic reticulum. This is identified as a typical structural feature of the basal ectoplasmic specialization [95]. There are three types of tight junction transmembrane proteins that are associated with the BTB: claudins, occludins (in rats), and junctional adhesion molecules [96–98]. The two AJ proteins associated with the BTB are the classic cadherins and nectin-2 [99, 100]. Both the TJ and AJ proteins become linked to actin filaments by adaptors, strengthening the BTB to ensure separation of spermatogonia and preleptotene/leptotene spermatocytes from the spermatocytes that are completing meiosis in the adluminal compartment [101]. In this way, the BTB serves as both an immunological and reproductive toxicant barrier by separating postmeiotic spermatocytes from systemic circulation. During spermatogenesis, preleptotene and leptotene spermatocytes pass through the dynamic BTB; when this process is disrupted, germ cells cannot develop or differentiate normally.

Because of the intricate hormonal events required to initiate puberty and the transition to sexual maturity, this developmental stage is anticipated to be sensitive to alteration by EDCs. Yu *et al*. [102] observed severe reproductive histopathology after the administration of vinclozolin, a systemic dicarboximide fungicide with antiandrogenic activity, to male rats during the pubertal period. Rats dosed with 100 mg/kg/day displayed a decrease in the weight of accessory sex organs such as epididymal and seminal vesicle weights. Histopathology of rats dosed with 200 mg/kg/day included hypertrophy of Leydig cells, decreased prostate weight, and detached debris and sloughed germ cells in the caput epididymis, indicating a spermatogenic disorder in the testis. At the high dose of 300 mg/kg/day, an increase in serum testosterone was observed as well. Blystone *et al*. [103] saw a similar effect following administration of iprodione, another antiandrogenic fungicide, with decreases in prostate and seminal vesicle weights at 100 mg/kg/day. This study also consisted of a pubertal mixture study, examining the effect of an iprodione and vinclozolin coexposure. Administration of both compounds together produced an additive effect on androgen-sensitive endpoints. Interestingly, however, iprodione appeared to have an inhibitory effect on the vinclozolin-induced increase in testosterone. Administration of vinclozolin and other EDCs to pubertal male rats appears to impair normal differentiation of reproductive organs during puberty, possibly by disturbing the precise endogenous endocrine landscape.

The end of puberty marks male sexual maturity, at which point reproductive organs and accessory sex glands have reached their adult state. The major components of the adult male reproductive system, including the testes, the epididymis, and the prostate, are described below.

Biology and Toxicology of the Adult Male Reproductive Tract

Structure and Function of the Adult Male Reproductive System

The testes are firm, oval shaped glands found in the mammalian scrotum. The adult testis is a complex organ whose two major functions, spermatogenesis and steroid hormone production, are highly dependent upon the coordinated regulation of interacting cell types, namely, Leydig cells, peritubular myoid cells, Sertoli cells, and germ cells (Fig. 2). Structurally, the testes are covered by the tunica albuginea,

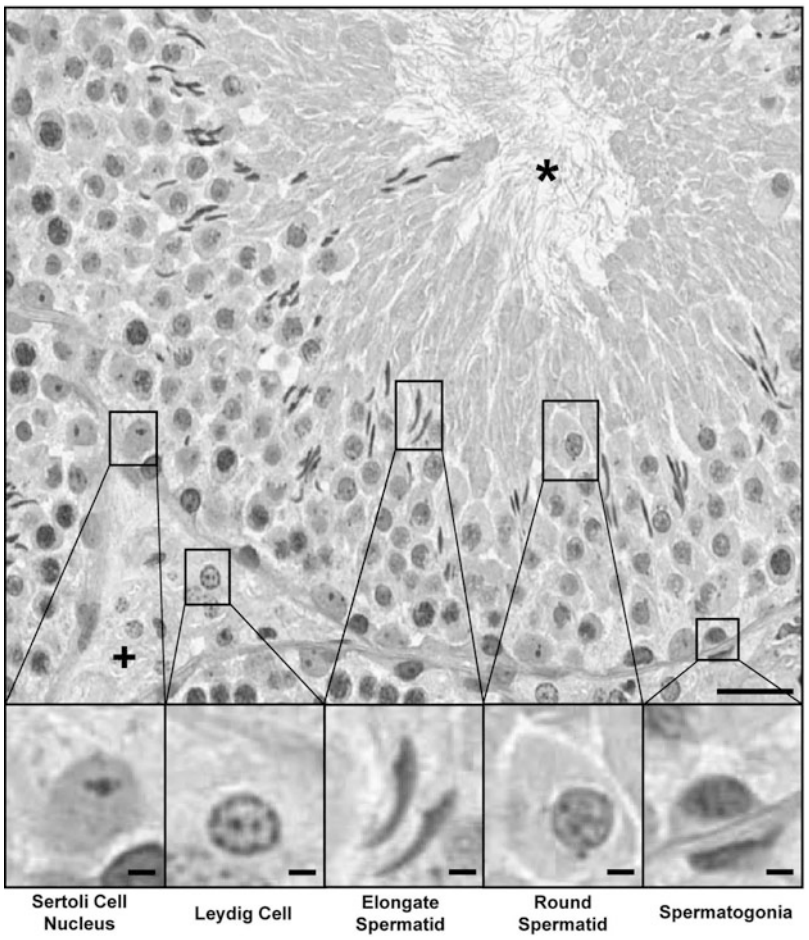


Fig. 2 Cross section of an adult rat seminiferous tubule demonstrating normal spermatogenesis. The lumen of the seminiferous tubule (*asterisks*), and interstitial space (*plus*) are identified (bar = 25 μm). Along the *bottom* of the figure, the individual cell types are labeled and magnified to illustrate their morphology (bar = 3 μm). H&E staining

a layer of connective tissue. The testis contains two major compartments—the seminiferous tubules and the interstitial spaces. Blood vessels, lymphatic vessels, and Leydig cells are found in the interstitial space; as the interstitial cells are directly adjacent to the seminiferous tubules, Leydig cells are responsible for the production and release of testosterone. The seminiferous tubules, covered by peritubular myoid cells, are finely coiled tubes organized in loops throughout the organ that connect to the excurrent duct system and contain both Sertoli cells and germ cells. The Sertoli cells form the BTB and act as "nurse" cells that provide the nutrients and environment necessary for spermatogenesis. They also phagocytose both apoptotic germ cells during normal spermatogenesis and the residual spermatid cytoplasm during spermiogenesis [104]. Additionally, Sertoli cells secrete fluid that forms a tubular lumen and transports sperm to the epididymis. Typically, Sertoli cells do not proliferate once they are fully differentiated, so by the time spermatogenesis has begun, the Sertoli cell population is fixed in number [104]. Spermatogonial germ cells line the basement membrane of the seminiferous tubules and act as sperm progenitor cells; they maintain close contact with the Sertoli cells and move inward toward the lumen as they proliferate and differentiate. The seminiferous tubules converge at the rete testis; from here, the immature sperm formed in the testis are transported through the efferent ducts to the caput epididymis.

The testes are critical end-organ components of an endocrine feedback system responsible for testosterone synthesis. Testosterone is produced by a highly regulated pathway that begins with the secretion of GnRH by the hypothalamus. GnRH stimulates the anterior pituitary to secrete LH and FSH [104]. LH receptors on Leydig cells are sensitive to FSH-induced upregulation, making the cells more responsive to LH. Leydig cells respond to LH stimulation by enhancing cholesterol desmolase activity, which converts cholesterol to pregnenolone, leading to testosterone synthesis and secretion [104]. Testosterone is necessary for normal spermatogenesis by activating pathways in Sertoli cells that promote differentiation of spermatogonia.

Spermatogenesis occurs in three phases: spermatogoniogenesis, meiosis, and spermiogenesis [105]. During spermatogoniogenesis, the spermatogonia in the basal compartment undergo multiple mitoses to build a large population of cells for subsequent meiosis and differentiation. In humans, there are three subtypes of spermatogonia: type A(d) cells, type A(p) cells, and type B cells [104]. Type A(d) cells have dark nuclei and replicate to ensure a constant supply of spermatogonia to fuel spermatogenesis. Type A (p) cells have pale nuclei and divide by mitosis to produce type B cells. Type B cells divide to give rise to primary spermatocytes. From here, each primary spermatocyte moves into the adluminal compartment of the seminiferous tubule, where it enters the second phase of spermatogenesis by undergoing meiosis I to produce two secondary spermatocytes [104]. These maturational steps are sources of genetic variation, such as chromosomal crossover or random inclusion of either parental chromosome, increasing the genetic variability of the gamete. Secondary spermatocytes rapidly enter meiosis II and divide, yielding haploid spermatids. These spermatids differentiate into spermatozoa through a process called spermiogenesis, during which the spermatids begin to grow a tail and develop a thickened midpiece where the mitochondria gather and form an axoneme [104]. In addition, the haploid nucleus is streamlined, and the Golgi

apparatus surrounds the condensed nucleus, forming the acrosome. The nuclear cytoplasm is eliminated and chromatin compaction occurs, whereby the somatic and testis-specific histones are replaced with transition proteins and protamines [106]. The male germ cell differentiation program requires an array of finely tuned levels of gene regulation [106]. Chromatin compaction causes the cessation of transcription in elongating spermatids. However, high levels of mRNAs are found in round spermatids before transcriptional arrest and are stored in a stable form in preparation for their translation during the later stages of spermiogenesis [106]. mRNA storage and subsequent translational activation are very important in regulating the synthesis of many sperm proteins. Maturation takes place under the influence of testosterone. The excess cytoplasm of the spermatids forms into residual bodies and is phagocytosed by surrounding Sertoli cells. The resulting spermatozoa, mature but lacking motility, are released from the Sertoli cells into the lumen of the seminiferous tubule in a process called spermiation [104]. The nonmotile spermatozoa are transported to the epididymis by peristaltic contraction, where they gain motility and become capable of fertilization. Mature sperm consist of a haploid nucleus, a propulsion system, and an acrosomal sac of enzymes that enable the nucleus to enter the oocyte.

Sperm were originally regarded as a vessel for the transportation of the male genome to the oocyte, devoid of translational activity (due to their lack of cytoplasm and ribosomal RNAs); the oocyte, on the other hand, was believed to be responsible for producing all of the mRNA and proteins necessary for fertilization and embryogenesis. However, recent data suggest that sperm play a greater role in this process than originally believed; in addition to their genome, sperm transmit mRNA and provide the oocyte with vital organellar and male-specific proteomic components. At least 5,000 different mRNA transcripts are currently known to exist in sperm [107]. These characterized sperm transcripts are involved in cell signaling/communication, cell division, gene/protein expression, metabolism, cell structure and motility, and organism defense [108]. Many functions have been proposed for these sperm transcripts, including roles in sperm structure and stress response, *de novo* translational replacement of degraded proteins, oocyte fertilization, embryogenesis/morphogenesis, and epigenetic regulation and establishment/maintenance of the parental imprint [108, 109]. At present, it is understood that the quantity and types of mRNA transcripts may indicate the state of spermatogenesis [107].

Epididymis

The epididymis is a complex coiled tube that connects the testis to the vas deferens and functions to transport, nurture, and mature the sperm. Its structure is designed to facilitate these processes both hormonally and physically. The epididymis is divided into three sections, the caput, the corpus, and the cauda, each of which plays a role in the development of the sperm as it travels through the organ. Secretory products produced by the epididymal epithelium lead to numerous functional changes of the sperm that include acquisition of motility and increased capacity to fertilize [110, 111].

Prostate

The prostate surrounds the urethra just below the bladder; this location allows for the control of urine flow during ejaculation. The prostate is a complex organ composed of both glandular and nonglandular tissues encased in a fibromuscular capsule. The two major regions of the prostate, the peripheral zone and the central zone, compose about 70% and 25% of the glandular mass, respectively [112], while a smaller transition zone consists of about 5–10% of the prostatic glandular tissue. The ducts from the different glandular regions drain into the urethra at different points. The prostate stores and secretes a milky white, slightly alkaline fluid (pH 7.29) that combines with spermatozoa and other secretions during ejaculation. The prostatic fluid contributes about 25–30% of the volume of the ejaculate and, along with secretions from the seminal vesicles, neutralizes the acidity of the vaginal tract to increase sperm survival and facilitate fertilization. Prostatic fluid also contains prostate-specific antigen (PSA), which further liquefies the semen after ejaculation, improving sperm motility [113].

Model Adult Testicular Toxicants

2,5-Hexanedione (HD, Fig. 3a) is the active metabolite of the common industrial solvent, *n*-hexane. Although the most significant exposures occur in occupational settings, humans are ubiquitously exposed to low levels of *n*-hexane as a chemical component of gasoline [114]. HD is both a neuronal and testicular toxicant with clear neurotoxic clinical manifestations and subtle indications of testicular injury. Animal studies indicate that HD targets Sertoli cell microtubule assembly by the induction of tubulin cross-linking both *in vitro* and *in vivo* [114]. HD exposure causes altered microtubule-dependent transport in Sertoli cells and disturbs the germ cell niche by impeding seminiferous tubule fluid secretion. Disruption of the Sertoli cell microenvironment stimulates germ cell apoptosis and ultimately results in testicular atrophy [114].

1,2-Dibromo-3-chloropropane (DBCP, Fig. 3b) is a nematocide that has been shown to reduce fertility and induce sterility in humans exposed in occupational settings [115]. DBCP was used from 1950 to 1979, at which time it was banned in the United States due to the adverse health effects that had been identified a few years earlier. In 1977, workers at the Dow chemical company complained of deleterious health effects in connection with the chemical, and indeed, published laboratory findings of these workers showed that they suffered from oligospermia, azoospermia, and elevated hormone levels [115, 116]. Animal experiments have also revealed the testicular toxicity of DBCP; exposed rats display altered seminiferous tubule morphology, malformations of the sperm, and reduced sperm counts [117]. Studies also indicate that DNA is the subcellular target of this toxicant. DBCP can be converted to reactive metabolites both by cytochrome P450-dependent monooxygenase (CYP)- and glutathione *S*-transferase (GST)-dependent pathways [118], and these resulting metabolites can induce single-strand breaks in DNA.

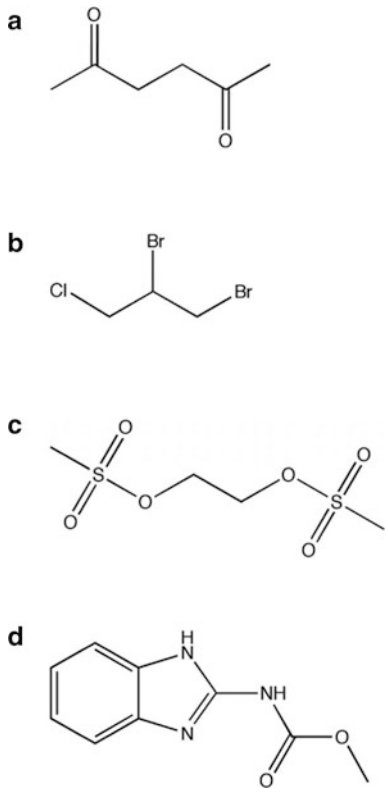


Fig. 3 Chemical structures of model testicular toxicants: (**a**) 2,5-hexanedione (HD), (**b**) 1,2-dibromo-3-chloropropane (DBCP), (**c**) ethylene-1,2-dimethanesulfonate (EDS), and (**d**) carbendazim (CBZ)

The amount of GSTs in the spermatogenic cell types increases with spermatogenic cell development [118], which may indicate a higher potential to activate DBCP in germ cells at later stages of development. In addition, cells in S-phase are more susceptible to DBCP-induced apoptosis than cells in the growth phases [118]. In accordance with the model, proliferating and differentiating spermatogenic cells (e.g., round spermatids) are the cells most sensitive to DBCP-induced apoptosis. Because round spermatids have less compacted DNA than elongating/elongated spermatids, the DNA in these cells is a more accessible target.

Ethylene-1,2-dimethanesulfonate (EDS, Fig. 3c) is a toxicant that selectively and temporarily destroys the adult Leydig cell population through apoptosis via the Fas/FasL pathway [119]. An intraperitoneal (IP) injection of 75 mg/kg in adult rats decreases testicular and serum testosterone and increases the pituitary secretion of LH and FSH [120]. Overall, EDS induces a depletion of germ cells in the seminiferous epithelium. Leydig cells disappear from the interstitial space by 7 days post-IP injection, as evidenced by histological examination. Additionally, there is a decrease in elongating (step 9–13) spermatids in late stage seminiferous tubules (IX–XIII) [120], although the early stages remain intact. Single new Leydig cells with characteristics of progenitor type Leydig cells are observed at 2 weeks postexposure. There is

also a loss of all stages of elongated spermatids and germ cell sloughing into the lumen [120]. At 3 weeks postexposure, there is an expansion of immature Leydig cells and signs of recovering spermatogenesis including the presence of elongated spermatids in late but not early stages [120]. At 5 weeks postexposure, mature adult type Leydig cells are visible in the interstitial space. The late stages are almost completely recovered (there is evidence of all germ cell types), although there are still no elongated spermatids found in early stages [120]. By 7 weeks postexposure, the Leydig cell population has resumed a normal histological appearance. Spermatogenesis appears recovered in most of the tubules, although some of tubules still contain only Sertoli cells [120]. There is a close relationship between germ cell and Leydig cell changes; on the subcellular level, it is believed that the EDS-induced testosterone reduction causes apoptosis of germ cells (particularly of haploid germ cells) and the temporary arrest of spermatogenesis [120].

Carbendazim (CBZ, Fig. 3d) is the active metabolite of benomyl, a benzimidazole fungicide used to prevent and eliminate fungal plant diseases [121, 122]. Mammals are exposed to CBZ orally, and it is readily absorbed and metabolized. Overall, CBZ has low acute toxicity but has many negative effects on the male reproductive system [121, 122]. CBZ is a Sertoli cell toxicant that targets those cells and inhibits microtubule polymerization by binding to the β-tubulin subunit of the tubulin heterodimer. This ultimately decreases the rate and stability of microtubule assembly [121]. Acute exposure of adult male Fisher 344 rats to CBZ results in increased testis weights and seminiferous tubule diameters as well as increased rates of germ cell sloughing, retained spermatid heads, and apoptotic germ cells 1 h postexposure in a dose-dependent manner [121]. Subchronic exposure of adult male Wistar rats to CBZ resulted in many histopathological changes of the testis including atrophic seminiferous tubules, decreased germ cells, and increased sloughing in a dose-dependent manner [122]. These rats had smaller testes and decreased epididymal sperm counts and motility and mating studies demonstrated a dose-dependent decreased fertility index [122]. LH was the only hormone significantly altered in the high-dose group [122]. Flow cytometric analysis of the testicular tissue indicated an interference of the spermatogenic process, showing a dose-dependent increase in primary spermatocytes and a decrease in the number of spermatogonia, spermatids, and DNA-synthesizing cells [122]. Overall, CBZ disrupts proper Sertoli cell function and spermatogenesis and ultimately reduces fertility in male rats [121, 122].

Exogenous Hormones

Anabolic-androgenic steroids (AASs) are synthetic analogs of testosterone. AASs are used to treat a variety of conditions including refractory anemia, hereditary angioedema, breast cancer, and starvation states [123]. At super-therapeutic doses (10×–100×), AASs are known to improve athletic ability and increase muscle mass. However, using these drugs for off-market purposes has negative effects on both human and animal physiology. In humans, AAS users have reported

alterations in libido, changes in mood, reduced testis volume, acne, and gynecomastia [124]. In adult rats exposed to the AAS nandrolone decanoate for 2 weeks (followed by a 2-week recovery period), decreases were reported for mean testis volume, length of seminiferous tubules, sperm count, and motility [123]. Rats exposed to a cocktail of AASs (nandrolone decanoate, metenolone acetate, and dromostanolone) subcutaneously once a week for 6 weeks followed by a 4-week recovery period and an additional 6 weeks of treatment had increases in serum testosterone and serum DHT [125] and showed signs of hair loss, aggressive behavior, and self-inflicted tail wounds. There was observable physical damage to the heart, testis, and adrenal gland, and histology showed reduced numbers of Sertoli cells, Leydig cells, and spermatozoa [125]. Exogenous administration of synthetic testosterone analogs resulted in negative feedback on the HPG axis and an inhibition of FSH and LH, which ultimately led to an inability to maintain spermatogenesis due to hypogonadotropic hypogonadism and testicular atrophy [123].

Protection from Testicular Injury

Ubiquitin marks aberrant proteins for 26S proteasome degradation, and it is believed that a mutated form of ubiquitin (K48R) offers protection from testicular injury in both acute (experimental cryptorchidism) and chronic (aging) cases. This is true for mice with the K48R ubiquitin mutation, which are resistant to the effects of acute and chronic testicular injury [126]. Experimental cryptorchidism increases testicular heat stress, leading to a cascade of events that include altered protein degradation, germ cell loss, and testicular atrophy; aging can also lead to germ cell loss and atrophy of the testes. When compared to wild-type mice, ubiquitin K48R mutant mice have greater testis weights after both types of injury [126]. Testis cross sections of experimentally cryptorchid animals indicate that the average number of germ cells per seminiferous tubule is greater in ubiquitin K48R mutant mice than in their wild-type counterparts. The seminiferous tubules of aged mutant mice have larger diameters, with a greater number of germ cells, than wild-type mice, indicating that the K48R mutation does indeed serve to protect against the testicular injury of aging. It was also observed that the mutation protected blood vessels in the interstitial space from becoming enlarged with hyaline material accumulations [126].

Testicular atrophy can be the result of a variety of insults, including environmental toxicant exposure, chemotherapy, irradiation, and aging [127]. It is believed that treating males with GnRH analogs after testicular insult can prevent or reverse atrophy [127] because GnRH is a peptide hormone that induces the release of LH and FSH from the anterior pituitary. GnRH analogs (agonists and antagonists) are used to treat many clinical conditions, including prostate cancer, because they offer reversible medical castration with effects similar to that of orchiectomy [128]. GnRH agonists directly stimulate the production of LH and FSH which ultimately leads to increased production of testosterone. Despite this initial surge of testosterone production, prolonged occupation of pituitary LH receptors results in an overall

decrease in testosterone. Conversely, GnRH antagonists directly inhibit the GnRH receptor and decrease levels of LH, FSH, and testosterone [128].

As previously mentioned, radiation exposure induces testicular atrophy by depleting the germ cell population. The seminiferous tubules of irradiated rats contain normal undifferentiated type A spermatogonia; rather than differentiating, these cells become apoptotic, and the number of cells remains constant [127]. In addition, irradiated rats have elevated levels of intratesticular testosterone and systemic FSH compared to controls. When a GnRH analog is administered immediately after irradiation, the seminiferous tubules maintain spermatogonial differentiation up to the round spermatids. After a recovery period allows increases in testosterone and FSH levels, the testes of irradiated rats treated with GnRH resume normal spermatogenesis for at least 12 weeks. It appears as though the transient suppression of intratesticular testosterone and FSH prevents the persistence of the radiation-induced testicular atrophy. Spermatogenic recovery is disrupted when rats are given exogenous testosterone simultaneously with GnRH treatment [127]; FSH levels are also inversely correlated with recovery. Therefore, under normal conditions, testosterone is required for spermatogenesis; however, in irradiated male rats, elevated intratesticular testosterone and FSH inhibit early spermatogonial differentiation [127].

Similarly, the testes of rats treated with the environmental toxicant 2,5-hexanedione (HD, Fig. 3a) (1%) for 3–5 weeks are atrophic 12 weeks after the start of exposure and remain so for at least 70 weeks postexposure [127]. Histopathological examination reveals that less than 1% of seminiferous tubules contain germ cells more advanced than type A spermatogonia after this protracted HD exposure [127]. In animals treated with a GnRH agonist immediately after 3–5 weeks of HD exposure, 90% of seminiferous tubules contained advanced germ cells and greater than 80% contained elongated spermatids. Temporarily eliminating testosterone by ablating HD-treated rats with multiple injections of EDS only temporarily increased spermatogenic recovery, indicating that suppression of testosterone alone is not enough to reverse testicular atrophy [127]. Ablating HD-treated rats with one injection of EDS followed by GnRH treatment (3×) resulted in no spermatogenic recovery. However, HD-treated rats treated 3× with GnRH and no Leydig cell ablation did lead to the restoration of spermatogenesis. These results suggest that Leydig cell factors are important in spermatogenic recovery of the atrophic testes [127].

Finally, similar experiments were conducted with aged Brown Norway rats. These animals had decreased serum and testicular testosterone, which suggests that an increase in testicular testosterone is not required for the maintenance of atrophy. Treatment of aged rats with GnRH (3×) led to a modest increase in spermatogenesis [129].

Sperm Biomarkers of Spermatogenic Abnormalities

An emerging research area is that of human sperm mRNAs as potential biomarkers of fertility. Ostermeier *et al.* found that a genetic fingerprint of normal fertile men can be generated from mRNA present in sperm [107] and that studying ejaculated

sperm is a convenient method for investigating testis-specific infertility. Wang *et al.* used microarray technology to analyze gene expression differences between testis-specific and sperm-specific genes in fertile men [130]. By examining the expression of five sperm motility-related genes identified in this profile by reverse transcriptase polymerase chain reaction (RT-PCR), they found the expression of two genes, TXP1 and LDHC, to be significantly altered between normal and motility-impaired semen samples. Again, their results indicated that clinical assessments of sperm quality can be made from differential sperm mRNA content patterns. Additionally, human studies have indicated that abnormal protamine ratios exist in infertile men, and functional evidence has demonstrated that male protamine knockout mice are infertile [131, 132]. With this in mind, Steger *et al.* investigated the potential use of protamine ratios and Bcl2 expression as biomarkers of infertility using RT-PCR [133]. They found aberrant protamine ratios and increased mRNA expression of Bcl2 in ejaculates and in testicular biopsies of infertile men compared to controls. These results encourage the use of mRNA expression levels as predictive biomarkers of fertility status. As of yet, only one laboratory has focused on identifying biomarkers of fertility in animal models; Klinefelter *et al.* have studied expression of SP22, a sperm membrane protein, and its correlation with caudal epididymal sperm fertility after exposure to testicular and epididymal toxicants [134]. In addition, they have confirmed that postmeiotic germ cells express a testis-specific SP22 transcript. The same group is currently examining the potential use of this protein as a diagnostic marker of human infertility [134].

Epigenetic mechanisms, and specifically DNA methylation, play an important role in regulating genes during development. Methylation is a heritable yet reversible epigenetic mark that influences gene expression without altering the underlying DNA sequence. Mammalian DNA is methylated at the 5-position of cytosine residues primarily within CpG dinucleotides; this reaction is catalyzed by a family of DNA methyltransferases. Methylation occurs almost exclusively at CpG dinucleotides in the CpG islands located in the promoter region of genes [135]. DNA methylation modifies the function of the mammalian genome and typically results in repression of gene expression. DNA methylation is essential for normal development [136, 137]; the epigenetic reprogramming that occurs during development may be a sensitive window for disruption of the epigenome. It has been suggested that alterations in the epigenetic reprogramming processes during development can lead to adult-onset disease [13].

In the developing embryo, primordial germ cells become demethylated as they migrate along the genital ridge toward the fetal gonad. During gamete maturation, the methylation profile is reestablished in the germ line [138], resulting in a pattern of DNA methylation that reflects both inherited imprints and environmental conditions. CpG dinucleotides are underrepresented in the genome but overrepresented in promoter regions. Hypomethylated promoter regions establish an open chromatin structure that allows for the initiation of gene transcription, while hypermethylated promoter regions lead to a closed chromatin structure, blocking transcription factor binding and silencing gene expression [139]. In addition to regulating gene expression, DNA methylation silences repetitive elements and is important for the stability of the mammalian genome.

Both human and animal studies have shown that abnormal methylation patterns affect fertility. Houshdaran *et al*. examined the global methylation pattern of sperm in semen samples isolated from male members of 69 couples referred for infertility analysis and found that in poor quality sperm, the methylation state of numerous sequences was elevated in the DNA [140]. They hypothesized that the mechanism behind the epigenetic change may be aberrant erasure of DNA methylation during epigenetic reprogramming of the male germ line [140]. In addition, Pathak *et al*. examined the effects of tamoxifen exposure on DNA methylation patterning in rat spermatozoa [141]. The authors measured global sperm DNA methylation, the methylation state of the Igf2-H19 imprinting control region (ICR), and embryo postimplantation loss. Although no changes in global methylation were seen, methylation was reduced at the Igf2-H19 ICR. Mating experiments showed a significant increase in postimplantation loss, which positively correlated with the reduced ICR methylation. The authors suggest that errors in paternal imprints could affect embryo development and that methylation patterns could be useful as biomarkers for evaluating male fertility [141].

Biology and Toxicology of the Aging Male Reproductive Tract

After a male reaches sexual maturity, his reproductive system remains fertile and unchanged until he experiences either testicular injury or reduced reproductive system function as a result of age. Aging of the male reproductive tract brings about many changes at the regulatory, molecular, and cellular levels, which, unlike changes during fetal development, are not genetically programmed [138]. Some characteristic morphological changes of the testis associated with age are decreased Leydig cell numbers, leading to a decrease in testosterone production, arteriosclerotic lesions, and thickening of the tunica albuginea [142, 143]. Male aging has also been associated with decremental changes in HPG axis function, with an amplitude decrease in GnRH and LH and an increase in pulse frequency, ultimately leading to a decrease in the production of testosterone [144].

Because these age-related changes have also been identified in rodents, rats represent a good model organism for studying the aging reproductive tract. Some rat strains are more useful than others; for example, the Brown Norway rat model may more closely resemble human reproductive tract aging and is preferred over both the Sprague–Dawley and Wistar strains for several reasons. First, the Sprague–Dawleys and Wistars have health problems that generally accompany aging (such as pituitary adenomas, obesity, and testicular tumors), a fact that leads to difficulty differentiating between age-related and disease-related defects in the male reproductive tract [145]. Second, the average lifespan of a Brown Norway rat is 36 months, compared to the 27 months that a Sprague–Dawley rat is expected to survive [145]. Brown Norway rats also offer a larger window for studying the age-associated decrease in testosterone production in the testis (which includes both serum and intratesticular levels); this testosterone decline occurs over an extended

time of months 18–30 in Brown Norway rats but only between months 21 and 24 in the Sprague–Dawley strain [145]. In addition to having lower testosterone, Brown Norway rats have decreased Sertoli cell function and numbers and a marked reduction in seminiferous tubule volume and contents [146]. In contrast to the Sprague–Dawley and Wistar strains, Brown Norway rats live for more than a year after these changes occur in the testis and generally remain healthy throughout the entire period of testicular aging [145]. For these reasons, the Brown Norway strain is the preferred rat model for studying human reproductive tract aging [145–147].

Research using Brown Norway rats has suggested that the age-related reduction in steroidogenesis is due not to a decrease in Leydig cell number or a change in Leydig cell responsiveness to LH [147] but rather to atrophic changes in Leydig cell size and organelle content with aging. Because testosterone production is a function of Leydig cell size [147, 148], these changes lead to dysfunction of the steroidogenic process. Decreases in testicular weight, sperm concentration, total sperm counts, plasma testosterone, LH, and inhibin begin at 15 months [146]. Conversely, the proportion of regressed testes, plasma FSH levels, and germ cell loss via apoptosis increases with aging [146]. In 15-month-old males, 6 months of LH replacement therapy did not decrease or delay any age-related effects on the testis [146]. However, when 18-month-old males were treated with LH, thyroxine (T_4), or a combination of both for 4 weeks, an increase in steroidogenic ability was observed [148]. The same study showed that with a daily dose of 24 + 5 μg dose of LH + T_4, Leydig cells can recover 100% of their volume and their steroidogenic ability [148]. These results indicate that changes in LH and thyroxine levels in serum influence the decrease in Leydig cell volume and the loss of steroidogenic potential associated with aging [148]. Decreased thyroid hormones and LH result (both directly and indirectly) from the aging of the pituitary gland, suggesting that testicular aging is correlated with pituitary aging [148].

Protection from the effects of aging-induced testicular senescence is conferred by alterations in the function of the ubiquitin system, as described above. In addition, using the Brown Norway rat model, GnRH analog administration provides modest protection against testicular degeneration associated with aging in this model.

There is evidence that sensitivity to toxicants may differ in old age. For many drugs, these differences exist due to age-related changes in the number of receptors available, including steroid receptors, which have been shown to decrease with age [149]. Importantly, many tissues also develop a greater sensitivity to exogenous insults over time, so that the aged tissue is more susceptible to toxicants than its younger counterpart.

Distribution of the chemical in the organism can also influence relative toxicity; with age, it can change in two major ways: first, the reduction in plasma albumin that is common with aging can lead to an increase in the amount of free compound that is available in the body, increasing its toxicity. Second, an overall decrease in lean body mass among the elderly (in which the percentage of adipose tissue increases and body water decreases) changes the patterns of distribution such that water-soluble compounds have a smaller volume of distribution and lipophilic

compounds, such as PCBs, have a greater volume of distribution [149]. Rates of metabolism and excretion also vary with age, as shown for TCDD comparing juvenile, adult, and senescent rats [150]. Finally, decreased renal function in the aged can lead to a higher concentration of a chemical in the body for a longer period of time, increasing the risk of toxicity [149].

Complex Exposures and Low-Dose Effects

Humans are exposed to many of the approximately 70,000 commercial and industrial chemicals currently in use on a daily basis. Because exposure to any single toxicant rarely occurs independently of other chemical exposures, it is prudent to examine toxicant effects in the context of complex mixtures or coexposures that more adequately represent the true experience of exposure. In 1939, Bliss emphasized the importance of studying mixtures when he said that "the effect of the mixture cannot be assessed from that of individual ingredients, but depends upon knowledge of their combined toxicity when used in different proportions. One component synergizes or antagonizes the other" [151]. By 1996, there had been enough cases of complex chemical interactions that the Food Quality Protection Act (FQPA) was passed, requiring cumulative risk testing of chemicals with similar mechanisms of toxicity. While it is possible that chemicals with different mechanisms of toxicity act independently and that the effect of their coexposures could be simply additive, when chemicals target the same organ system interactions among the cell types targeted within that organ may lead to complex interactive effects. The interaction of chemicals with similar targets or mechanisms of toxicity can result in addition, synergism (the effect is greater than the additive effect of two or more chemicals) or antagonism (the effect is lower than the additive effect of the chemicals) [152, 153].

The developing male reproductive tract is particularly sensitive to toxicants, and especially to endocrine disruptors. This vulnerability of male reproductive development is illustrated in a study by Howdeshell *et al*., in which fetal male rats were exposed to a mixture of two plasticizers, di-(*n*-butyl) phthalate (DBP) and di-(2-ethylhexyl) phthalate (DEHP) [154]. Mixture effects on several reproductive endpoints were demonstrated, including morphologic malformations and disruption in fetal steroid hormone production and the expression of Insl3 and genes that are responsible for the production of steroid hormones. Howdeshell *et al*. discovered that the mixture of DBP and DEHP elicited dose-additive effects, with an increase in many reproductive malformations by 50% or more [154]. In another study, Rider *et al*. examined the effects of a mixture of seven antiandrogens (vinclozolin, procymidone, linuron, prochloraz, benzyl butyl phthalate, dibutyl phthalate, and DEHP) on the period of male sexual differentiation in rats [155]. It was found that with increasing doses of this mixture, male offspring of the exposed female rat dams had a dose-dependent reduction in anogenital distance, all of the male offspring had hypospadias, and there was an 80% incidence of epididymal agenesis and

undescended testes in the high dose-treated dams [155]. This mixture of seven antiandrogens was comprised of compounds that act via different mechanisms on the androgen signaling pathway; exposure to this mixture resulted in a dose-additive disruption of male rat reproductive tract differentiation [155]. When any of these endocrine disruptors are administered alone, the effects are far less detrimental than when they are administered as mixtures. Since we are almost never exposed to a single toxicant at a time, the dose additivity seen with antiandrogenic mixtures speaks to the larger issue of interactive effects in combined exposures.

There is emerging evidence that adult exposure to mixtures of environmental chemicals can have harmful effects on the fully developed reproductive system. Andric *et al*. performed *in vivo* and *in vitro* studies with Aroclor 1248, a PCB mixture of congeners, in order to identify the effects of antiandrogens on the adult male reproductive system [156]. Results suggest that exposure to Aroclor 1248 causes downregulation of testicular androgenesis through inhibition of the activity of 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase/lyase, and 17β-hydroxysteroid dehydrogenase [156]. Markelewicz *et al*. (2004) examined the mixture effects of two testicular toxicants, 2,5-hexanedione (HD) and carbendazim (CBZ) (Fig. 3), that act on the microtubule function of Sertoli cells [157]. They found that when coadministered to adult male rats, these toxicants acted together in a synergistic fashion to intensify testicular injury. Studies of mixed exposure effects in adult animals are just the beginning of an effort to understand mixtures and their effects on humans in order to inform risk assessment. Most concerning is that both mixtures of chemicals with similar and different molecular mechanisms of action are capable of producing additive or more than additive effects in adult males, whose reproductive systems are generally less vulnerable to toxicant exposure than those of developing fetal males. Because humans are predominantly exposed to combinations of chemicals on a daily basis, it is important that studies designed to evaluate the health effects of mixtures are conducted.

In addition to the complication of mixtures risk assessment, some researchers have called for a reevaluation of the monotonic dose relationship central to the field of toxicology. Nearly all toxicological studies have assumed an increased response with increasing dose (monotonic). Recently, nonmonotonic dose-response patterns with low doses showing a stimulatory effect followed by inhibition at higher doses have gained attention [158] (see chapter *Hormesis: Improving Predictions in the Low Dose Zone*, authored by Calabrese). This "hormesis" phenomenon has been found in a wide variety of plants and animals and is postulated to help protect the organism against subsequent stresses through beneficial responses of enhanced growth, survival, and enzyme activity, among others. However, not all low-dose effects may be advantageous. A 2001 peer review report from the National Toxicology Program (NTP) found examples of nonmonotonic dose responses following low-dose endocrine disruptor exposure, with adverse outcomes such as changes to serum hormones, prostate weight, and mammary gland effects, with some occurring below the previously established NOAEL [159].

While effects at low doses cannot be disregarded, the vast majority of toxicology studies to date have focused on high doses and specific endpoints, which may not be predictive of low-dose effects. Depending upon the endpoint considered, the line between adaptive *versus* adverse responses becomes blurred [160]. In published studies that presumably show low-dose effects, little is known of the potential mechanisms of such effects, and whether these effects extrapolate across life stages and species [161]. Until further mechanistic understanding emerges, acceptance of low-dose effects and their applicability to human health risk assessment will remain unclear.

Transgenerational Effects and the Testis

Genomic Imprinting During Normal Development

Normal development requires that both maternal and paternal genomes are properly expressed. While most genes are expressed from both parental alleles, imprinted genes represent a subset of genes that are expressed in a monoallelic manner due to the differential methylation profiles of the alleles inherited from each parent. These imprints are established during gametogenesis or later in embryogenesis and are essential for normal development; there are several diseases that can result from improper imprinting [162]. Approximately 100 imprinted genes have been identified in mammals [163]. Genome-wide epigenetic reprogramming during gametogenesis and early embryogenesis functions to erase the maternal and paternal imprints in germ-line cells and reestablish these imprints according to the sex of the individual. For example, males contain one set of chromosomes with male imprints and one set with female imprints, but when these chromosomes are passed on to the next generation, both Y-bearing and X-bearing sperm must be reprogrammed to contain male imprints [164].

A wave of demethylation occurs following fertilization in the preimplantation mouse embryo, during which most gametic methylation differences are removed. The sperm genome is actively demethylated within 4 h of fertilization, and the egg genome is passively demethylated during subsequent cleavage divisions after the two-cell stage [165, 166]. However, most imprinted genes in somatic cells escape this wave, retaining gametic methylation patterns that will remain intact throughout embryonic development in order for the imprint to translate into the monoallelic gene expression that is required for further embryonic development [167, 168]. Following erasure of methylation patterns, reprogramming or reacquisition of methylation patterns of the developing male gametes occurs in order to endow sperm with imprinted alleles. Epigenetic paternalization is an ongoing process that begins to be acquired before birth in the gonocytes or prospermatogonia between 15.5 and 18.5 days of gestation in the mouse, continues after birth in the spermatogenic cells as they undergo mitotic and meiotic division, and is complete by the pachytene phase of meiosis [169, 170].

Transgenerational Passage of Toxicant Exposure Effects

The reprogramming of DNA methylation that takes place in primordial germ cells is presumably acting to prevent the passage of DNA methylation defects from one generation to the next [169]. However, recent studies have raised concerns about the potential for exposures during pregnancy to cause germ-line effects on imprinted genes resulting in transgenerational passage of DNA methylation defects [171, 172]. Chemical effects on offspring due to *in utero* exposures can be defined either as multigenerational or transgenerational. An exposure is considered multigenerational if the changes are due to direct exposure, which could affect the F0 mother, the F1 embryo, and the F2 germ line. A transgenerational effect does not involve a direct exposure and is defined as the transmission of a phenotype between generations that persists even without additional chemical exposure. Since the germ line that generates the F2 generation is present during a F0 exposure, a F2 generation phenotype is not a transgenerational phenotype. Only if this phenotype is present in the F3 generation, the first generation that has not had direct exposure to the toxicant, can this be deemed a true transgenerational phenotype [173].

A number of studies have suggested that environmental chemicals, such as EDCs, promote a transgenerational phenotype due to embryonic or postnatal exposures through an epigenetic mechanism [172, 173]. Exposure to endocrine disruptors during the sensitive windows of testis development and epigenetic reprogramming has been hypothesized to permanently reprogram the methylation patterns of the germ line, resulting in the transgenerational transmission of an altered phenotype and further resulting in adult-onset disease states. The reasoning behind this hypothesis is that due to the high frequency of adult-onset diseases, and the low frequencies of DNA sequence mutations, an epigenetic mechanism that does not involve DNA sequence mutations underlies these adult-onset diseases due to early exposures [172, 173]. While many DNA methylation changes are not heritable, it is thought that imprinted genes maintain methylation pattern in a heritable manner, and it has been proposed that alterations in the methylation status of imprinted genes may be a mechanism promoting these disease states [173, 174].

Vinclozolin, an antiandrogenic fungicide, and methoxychlor, an estrogenic pesticide, are endocrine disruptors that have been studied extensively for their potential to induce transgenerational effects. These studies have indicated that a persistent alteration in the epigenome can occur following *in utero* exposures [13]. The initial study that investigated the transgenerational effects of vinclozolin-exposed gestating Sprague–Dawley rats to vinclozolin by daily intraperitoneal injection of 100 or 200 mg/kg from gestational days (gd) 8–14 [172]; breeding of these offspring continued for four generations. Analysis of the F1–F4 generations revealed persistent reproductive effects in the males. There was a slight decrease in epididymal sperm counts and motility and, most significantly, increased spermatogenic cell apoptosis at a frequency of >90%. This frequency did not decline between the F1 and F4 generations, suggesting an epigenetic transgenerational effect [172]. When the vinclozolin F2 generation males were outcrossed to wild-type untreated control

females, the male progeny exhibited a similar phenotype of decreased spermatogenic capacity and male infertility, suggesting that the transgenerational phenotype is transmitted through the male germ line, likely through epigenetic changes (because the developmental period used for the endocrine disruptor exposure was during the remethylation programming of the germ line). The authors then performed DNA methylation analysis of testicular tissue, focusing on the effects of vinclozolin on the total genome, and identified 25 different PCR products with altered DNA methylation patterns that are associated with the transgenerational phenotype [172].

Candidate DNA sequences with altered methylation were further investigated in caudal epididymal spermatozoa obtained from vinclozolin-exposed F3 rats [174]. Fifteen of these sequences were found to be hypermethylated, prompting gene expression analysis; this was performed with gd16 male testis tissue in order to correlate these methylation changes to gene expression alterations. Some of the genes exhibited decreased expression, as expected with hypermethylation, while others exhibited increased expression. These investigations were taken a step further, and the F1 to F4 generations were analyzed for the development of adult disease states including prostate disease, kidney disease, and testis abnormalities [175]. Vinclozolin appears to increase the disease prevalence; however, the low sample size gives reduced confidence in these findings [13]. The authors suggest an epigenetic cause but make no connection between epigenetic changes and the observations of increased adult disease prevalence. While some of these observations have been challenged [176, 177], the potential importance of epigenetic modifications producing transgenerational effects, and the specific example of vinclozolin, warrants further investigation.

Testis Germ Cell Tumors and Endocrine-Disrupting Chemicals

Testicular germ cell tumors (TGCT) are most common among young men between the ages of 15 and 34 years old and are the predominant testis tumor type. They are characterized into subgroups based on histological characteristics: seminomas, nonseminomatous germ cell tumors, and spermatocytic seminomas [178]. In the United States, the occurrence of TGCT is slowly increasing [178]. Evidence suggests that men from European ancestry have a greater than fivefold incidence of TGCT compared to those of African descent, although the incidence of TGCT has been rising for black men as well [179]. The origins of TGCT are elusive; however, many investigators are exploring the possibility that fetal and early life EDC exposures can disrupt the critical hormonal balance during development and in turn contribute to the formation of TGCT later in life [180].

TGCT, one of the four proposed components of TDS, has both genetic and environmental aspects associated with its occurrence. It was found that men who have some form of gonadal dysgenesis (i.e., 45X/46XY) are more likely to develop TGCT in conjunction with other male reproductive abnormalities such as hypospadias and cryptorchidism [37, 181]. TGCT is also accompanied by additional

abnormalities within the seminiferous tubules [37]. Other strong associations include low birth weight [182, 183] or being born as a twin [184, 185]. These findings could be explained by altered nutritional intake or the presence of extra intrauterine estrogens, but this is still under investigation [182–185]. Finally, lifestyle represents an additional risk factor that could impact the developing fetus: smoking, occupation, maternal habits, and socioeconomic factors should all be considered when looking for the origins of TGCT [37, 181, 186].

Loss of fertility is associated with TGCT, although further studies are needed to fully characterize this relationship [187]. Precursor cells, known as carcinoma *in situ* (CIS) cells, are thought to give rise to TGCT [186]. CIS cells do not undergo differentiation and appear similar in morphology to fetal germ cells. They are characterized by the presence of placental-like alkaline phosphatase (PLAP) [188]. Although CIS cells are similar in appearance to fetal germ cells, they lack intercellular bridges and are often found along the edges of TGCT, with the exception of spermatocytic seminoma [187, 189]. There is no known mechanism for the formation of CIS cells; however, due to their morphologic similarity with fetal germ cells, a developmental origin is one potential mechanism for TGCT induction. Because dysregulation in secretion, signaling, production, transportation, and/or metabolism of steroidogenic pathways could impair germ cell differentiation and the surrounding cellular environment (potentially initiating carcinogenesis), research into the regulation of steroidogenic pathways and their associated hormones is becoming increasingly important. Investigation into these pathways has paved the way for further exploration into environmentally ubiquitous EDCs that may exert antiandrogenic effects during a critical period in development, thereby predisposing the fetus to reproductive abnormalities [189].

Exposure to Environmental Toxicants: A Potential Link to Testis Cancer?

The mechanisms behind the development of testis cancer are still unknown, but both environmental and lifestyle factors have been associated with its development. Although the strength of the association is unclear, some EDCs have been identified that may play a role in testis carcinogenesis, including certain types of persistent organic pollutants (i.e., organochlorine pesticides) [187], and exogenous estrogens [181].

Animal studies are helpful in assessing the effects of exogenous estrogens or antiandrogenic chemicals after *in utero* or early life exposures. Some examples of highly studied estrogenic compounds include DES, ethinyl estradiol, and bisphenol A (BPA), while antiandrogenic compounds include flutamide and vinclozolin. Results from animal studies have shown that *in utero* exposures to selected EDCs can lead to the development of hypospadias, cryptorchidism and reduction in sperm volume in the majority of the animals exposed, and in some severe cases the formation of Leydig cell tumors [37]. These initial studies provide some insight on the effects of early life exposures, but the animal models have significant limitations in terms of their relevance to human TGCT.

Phthalates, which have a wide application in commercial and industrial plastics, have been shown to alter male reproductive development by acting as antiandrogens within the fetal testis of rats, as described above [37]. Phthalates induce MNGs (Fig. 1) following a gestational exposure; MNGs have multiple nuclei that are contained within one cytoplasm and are postulated to be formed from the abnormal differentiation of germ cells. Although MNGs and CIS cells share a putative origin as developmentally dysgenetic germ-line cells, MNGs do not appear to lead to testicular cancer in rodent models [84, 190, 191].

Congenital Mouse Models for Exploring Mechanisms Behind Testicular Germ Cell Tumor Formation

Numerous efforts have been made to develop a rodent model that could replicate the characteristics of human testis cancer. Unfortunately, no animal model to date has formed the precursor CIS cells seen in the human. Furthermore, typical model animals, such as rodents, reach puberty much more quickly than humans, which could be a constraint when investigating the harmful developmental effects of various toxicants [192].

In the 1950s, using the 129/sv mouse substrain, Stevens generated the first mouse model that formed spontaneous testis tumors at a low frequency [193]. Spontaneous testis tumor formation is uncommon in other strains, suggesting that genetics play a key role. Mutations in the Ter gene led to tumor formation within weeks [194], that was related to a decrease in primordial germ cells initiated by the mutation [195]. A limitation of this mouse model is the formation of teratomas rather than seminomas, the more common TGCT type in humans.

An interesting tumor model more representative of a classic seminoma is found in mice that overexpress glial cell line-derived neurotrophic factor (GDNF), normally expressed by Sertoli cells. Tumors begin to form at approximately 1 year of age, with 56% of mice presenting bilateral tumors; human males, on the other hand, tend to exhibit unilateral tumors. Species-specific differences such as these emphasize the difficulties of extrapolating from animal models such as this to human TGCT [196].

Another important discovery in spontaneous tumor formation was made after investigating a deficiency in the p53 gene. Mouse models deficient in this gene form various types of spontaneous tumors, including testicular tumors [197, 198]. Mutation in this gene alters the normal cell-cycle control pathway and is the probable reason for carcinogenesis formation. In about 50% of animals studies, tumors presented as early as 10 months [199]. It is clear that different genetic backgrounds are capable of predisposing rodents to varying degrees of susceptibility to tumor formation; for example, mice with a 129/Sv background tend to develop a higher proportion of testis tumors (35%) than mice with a mixed C57BL/6 $\times$ 129/Sv background (9%) [200].

Overall, the existing animal models for TGCT have significant limitations, limiting the ability to explore the relationship between EDC exposure and testis germ cell tumor induction using such systems.

Prostate Diseases and Endocrine-Disrupting Chemicals

Development of the Prostate

Prostate development in humans begins between the 11th and 12th weeks of gestation with the development of solid ducts from the urogenital sinus [201]. Testosterone and other androgens send messages through the androgen receptors (AR) in the urogenital sinus mesenchyme (UGM) that promote growth of these initial duct buds into branched formations throughout the UGM; these further develop into a multifaceted arrangement of ducts. Unhindered communication between the epithelial and mesenchymal tissues is critical in the developing prostate. The mesenchyme promotes ductal growth by stimulating the production of androgen receptors within the epithelium, ensuring continued propagation of epithelial tissue and related secretory proteins. In response to mesenchymal signaling, epithelial cells promote smooth muscle differentiation, further contributing to growth of the mesenchyme [202]. Due to the reciprocal nature of this signaling, dysregulation is particularly detrimental and has been implicated in the development of disease states [203]. In humans, the initial phase of prostate development is completed at birth and resumes during puberty with increased levels of androgens. In rats and mice, prostate development follows a different trajectory; in these rodents, the prostate develops continuously from the end of fetal life until the beginning of adulthood [204].

The increasingly widespread incidence of prostate cancer has led to an explosion of research into its etiology. A dominant theory suggests that this disease, which manifests in adulthood, may have a basis in early development; for example, it is hypothesized that the use of exogenous hormones by pregnant women has led to an increase in developmental diseases in the prostate, testis, and breasts [205]. While the proposed mechanism remains unclear, data suggest that disruption of the natural hormonal balance of mother and fetus could result in a higher susceptibility to certain cancers.

Disruption of Normal Prostate Development by Exogenous Estrogen Exposure

Androgens were originally thought to play a central role in regulating normal and abnormal prostate progression. Androgens are well-known risk factors in certain age-related diseases, such as benign prostatic hypertrophy (BPH) and carcinoma [206];

however, recent evidence suggests that estrogens may also play a large role in the developing prostate and that disruption of the natural androgen/estrogen hormone balance could lead to abnormal growth [207]. Elevated endogenous estrogen during late gestation can induce spontaneous squamous metaplasia or extra layers of basal epithelium cells that degenerate rapidly after birth once estrogen levels return to normal. In aging adults, increased estradiol in conjunction with a decline in testosterone levels has been associated with the progression of prostatic diseases. Exogenous estrogens are ubiquitous in the environment, including in food (phytoestrogens) and its packaging. Natural exogenous estrogens, such as estradiol, are also found in foods, such as eggs, dairy, and meat products, and can be almost 10,000-fold more potent in binding to the estrogen receptor than phytoestrogens [208].

Two of the most commonly studied estrogens include DES and BPA. DES, a strong exogenous estrogen, was commonly used to treat pregnant women with threatened spontaneous abortions from the 1950s to the 1970s. In addition to functioning as an estrogen on the prostate, DES also has antiandrogenic effects by reducing the amount of LH that is secreted from the HPG axis (cf. above). This reduction in LH causes a decrease in testosterone production by the testes, which affects the androgen-dependent prostate [209]. It has been reported that in male stillbirths of mothers who ingested DES during pregnancy, there was a greater incidence of prostate abnormalities, including squamous metaplasia, enlarged ducts, and enlarged utricle [210]. Yonemura *et al*. xenotransplanted human fetal prostate tissue into athymic male nude mouse hosts and treated them with DES for 1 month. Implants in treated hosts developed severe squamous metaplasia, and injuries persisted even after cessation of DES treatment and reimplantation into a nontreated host [211]. Interestingly, the DES plus testosterone coexposed animals showed dysplastic lesions as well as the presence of carcinoma *in situ* cells [212]. These findings indicate that experiments concerning exogenous estrogen exposure during significant times in development are important to understanding disease progression in the prostate.

BPA, a ubiquitously present estrogen mimicking chemical, is a cross-linking agent used in the manufacture of polycarbonate plastics and epoxy resins (cf. above). Residual, unpolymerized BPA present in the plastic may leach into food or other media, particularly after being exposed to high temperatures or continual washes. BPA, however, does not bind with high affinity to the estrogen receptors as compared to endogenous estrogens such as estradiol [213]. Early studies using animals and the subcutaneous route of exposure (bypassing first-pass metabolism by the liver) showed that low-dose exposures to BPA during gestation could impact prostate growth and development by inducing an increase in cellular proliferation as well as the number of buds within the gland. These exposures also caused an apparent increase in adult prostate size without an accompanying morphological change [214].

To explore the importance of the timing of exposure to exogenous estrogens, Prins *et al*. developed a rodent model to determine whether "two hits" to endocrine-disrupting chemicals, such as BPA, might predispose to carcinoma formation in adulthood [214]. Briefly, low doses of either BPA (subcutaneous route) or estradiol were given to newborn male rats on neonatal days 1, 3, and 5 (one hit), critical

periods in development of the prostatic ducts. When the rats reached adulthood (day 90), a second treatment with estradiol was given by implanting silastic capsules that continuously released over 16 weeks (two hit). Prostate intraepithelial neoplasia (PIN) lesions, the precursors to prostate cancer, were found in 100% of rats in this two-hit model of combined exposure to BPA and estradiol. In comparison, rats given corn oil during the neonatal period followed by estradiol exposure in adulthood had only 40% incidence of PIN lesions. In addition to the presence of PIN lesions, an increase in epithelial proliferation and apoptosis was noted [213, 214]. This model underscores the potential significance of early life exposures to certain EDCs in predisposing to prostate disease later in life.

Summary

Since the term "endocrine-disrupting chemical" was introduced in 1993, animal studies have aimed to define the effects of these toxicants on the male reproductive system at each stage of development—fetal, pubertal, adult, and senescent. Because these chemicals mimic the roles of endogenous hormones, they can disrupt the delicate hormonal homeostasis that protects against testicular injury. The proposed TDS, consisting of cryptorchidism, hypospadias, impaired spermatogenesis, and TGCT, is a hypothesis that ties together potential downstream effects of early life endocrine disruption of the male reproductive tract.

There are several points during reproductive tract development that are particularly sensitive to endocrine-disrupting toxicants. In the fetal stage, hormones secreted by Sertoli cells and Leydig cells direct testis formation, testicular descent, spermatogenesis, and other important processes. It is during this stage that chemical insults could initiate disease processes that might not become visible until adulthood. Sexual maturation during puberty is initiated by GnRH from the HPG axis, increasing LH and FSH secretion from the pituitary gland. The resulting pubertal surge in testosterone initiates formation of seminiferous tubules and the migration and differentiation of germ cells into sperm. Formation of the BTB, an important immunological and reproductive toxicant barrier, occurs during the pubertal stage of development, making proper hormonal balance at this time critical for continued reproductive health. The end of puberty marks the beginning of adulthood and ultimately aging, when alterations in sperm quantity, morphology, and motility, and the occurrence of reproductive tract cancers (testis germ cell cancer and prostate adenocarcinoma) might be increased if early life developmental processes were disrupted.

Many decades of research aimed at identifying and quantifying the effects of toxicants on the male reproductive system have generated a wealth of new knowledge, including initial insights into complex exposures, low-dose responses, and transgenerational effects. Continuing research on male reprotoxicity, endocrine disruption, and the developmental origins of disease will yield new insights into the mechanisms responsible for disruptions of the male reproductive system throughout all life stages.

References

1. Barker DJ (1995) Fetal origins of coronary heart disease. Br Med J 311:171–174
2. Barker DJ (1997) Maternal nutrition, fetal nutrition, and disease in later life. Nutrition 13:807–813
3. Lumey LH, Stein AD, Kahn HS, Romijn JA (2009) Lipid profiles in middle-aged men and women after famine exposure during gestation: the Dutch Hunger Winter Families Study. Am J Clin Nutr 89:1737–1743
4. Heijmans BT, Tobi EW, Stein AD, Putter H, Blauw GJ, Susser ES, Slagboom PE, Lumey LH (2008) Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proc Natl Acad Sci USA 105:17046–17049
5. Painter RC, de Rooij SR, Bossuyt PM, Simmers TA, Osmond C, Barker DJ, Bleker OP, Roseboom TJ (2006) Early onset of coronary artery disease after prenatal exposure to the Dutch famine. Am J Clin Nutr 84:322–327; quiz 466–327
6. de Rooij SR, Painter RC, Phillips DI, Osmond C, Michels RP, Godsland IF, Bossuyt PM, Bleker OP, Roseboom TJ (2006) Impaired insulin secretion after prenatal exposure to the Dutch famine. Diabetes Care 29:1897–1901
7. Painter RC, Roseboom TJ, Bleker OP (2005) Prenatal exposure to the Dutch famine and disease in later life: an overview. Reprod Toxicol 20:345–352
8. Cooper RL (2009) Current developments in reproductive toxicity testing of pesticides. Reprod Toxicol 28:180–187
9. US-EPA (1996) Guidelines for reproductive toxicity risk assessment. U.S. Environmental Protection Agency, Office of Research and Development, Washington DC; EPA/630/R-96/009
10. US-EPA (1998) Health effects test guidelines, OPPTS 870.3800, reproduction and fertility effects. U.S. Environmental Protection Agency, Washington DC; EPA 712-C-98-208
11. Blazak WF, Treinen KA, Juniewicz PE (1993) Application of testicular sperm head counts in the assessment of male reproductive toxicity. In: Chapin RE, Heindel JJ (eds) Methods in toxicology: male reproductive toxicology. Academic, San Diego, CA, pp 86–94
12. Council NR (2007) Toxicity testing in the 21st century: a vision and a strategy. National Academy, Washington DC
13. Lebaron MJ, Rasoulpour RJ, Klapacz J, Ellis-Hutchings RG, Hollnagel HM, Gollapudi BB (2010) Epigenetics and chemical safety assessment. Mutat Res 705:83–95
14. Chapin RE, Stedman DB (2009) Endless possibilities: stem cells and the vision for toxicology testing in the 21st century. Toxicol Sci 112:17–22
15. Huston JM, Nation T, Balic A, Southwell BR (2009) The role of the gubernaculums in the descent and undescent of the testis. Ther Adv Urol 1:115–121
16. Wilson CA, Davies DC (2007) The control of sexual differentiation of the reproductive system and brain. Reproduction 133:331–359
17. Barsoum I, Yao HH (2006) The road to maleness: from testis to Wolffian duct. Trends Endocrinol Metab 17:223–228
18. Capel B (2000) The battle of the sexes. Mech Dev 92:89–103
19. Hannema SE, Hughes IA (2007) Regulation of Wolffian duct development. Horm Res 67: 142–151
20. Koopman P, Gubbay J, Vivian N, Goodfellow P, Lovell-Badge R (1991) Male development of chromosomally female mice transgenic for Sry. Nature 351:117–121
21. Gilbert SF (2006) Developmental biology. Sinauer Associates, Sunderland, MA
22. Lovell-Badge R (1992) The role of Sry in mammalian sex determination. Ciba Found Symp 165:162–179; discussion 179–182
23. Palmer SJ, Burgoyne PS (1991) *In situ* analysis of fetal, prepubertal and adult XX–XY chimaeric mouse testes: Sertoli cells are predominantly, but not exclusively, XY. Development 112:265–268

24. Merchant-Larios H, Moreno-Mendoza N (2001) Onset of sex differentiation: dialog between genes and cells. Arch Med Res 32:553–558
25. Sekido R, Bar I, Narvaez V, Penny G, Lovell-Badge R (2004) SOX9 is up-regulated by the transient expression of SRY specifically in Sertoli cell precursors. Dev Biol 274:271–279
26. DiNapoli L, Capel B (2008) SRY and the standoff in sex determination. Mol Endocrinol 22:1–9
27. Matsuzawa-Watanabe Y, Inoue J, Semba K (2003) Transcriptional activity of testis-determining factor SRY is modulated by the Wilms' tumor 1 gene product, WT1. Oncogene 22: 7900–7904
28. Luo X, Ikeda Y, Parker KL (1994) A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. Cell 77:481–490
29. Kreidberg JA, Sariola H, Loring JM, Maeda M, Pelletier J, Housman D, Jaenisch R (1993) WT-1 is required for early kidney development. Cell 74:679–691
30. Burgoyne PS, Buehr M, McLaren A (1988) XY follicle cells in ovaries of XX–XY female mouse chimaeras. Development 104:683–688
31. Yao HH, Whoriskey W, Capel B (2002) Desert Hedgehog/Patched 1 signaling specifies fetal Leydig cell fate in testis organogenesis. Genes Dev 16:1433–1440
32. Hughes IA, Acerini CL (2008) Factors controlling testis descent. Eur J Endocrinol 159 (Suppl 1):S75–S82
33. Creasy DM (2001) Pathogenesis of male reproductive toxicity. Toxicol Pathol 29:64–76
34. Klonisch T, Fowler PA, Hombach-Klonisch S (2004) Molecular and genetic regulation of testis descent and external genitalia development. Dev Biol 270:1–18
35. Overbeek PA, Gorlov IP, Sutherland RW, Houston JB, Harrison WR, Boettger-Tong HL, Bishop CE, Agoulnik AI (2001) A transgenic insertion causing cryptorchidism in mice. Genesis 30:26–35
36. Zimmermann S, Steding G, Emmen JM, Brinkmann AO, Nayernia K, Holstein AF, Engel W, Adham IM (1999) Targeted disruption of the Insl3 gene causes bilateral cryptorchidism. Mol Endocrinol 13:681–691
37. Skakkebaek NE, Rajpert-De Meyts E, Main KM (2001) Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. Hum Reprod 16:972–978
38. Foresta C, Zuccarello D, Garolla A, Ferlin A (2008) Role of hormones, genes, and environment in human cryptorchidism. Endocr Rev 29:560–580
39. Barthold JS (2008) Undescended testis: current theories of etiology. Curr Opin Urol 18: 395–400
40. Kalfa N, Philibert P, Sultan C (2009) Is hypospadias a genetic, endocrine or environmental disease, or still an unexplained malformation? Int J Androl 32:187–197
41. Krone N, Arlt W (2009) Genetics of congenital adrenal hyperplasia. Best Pract Res Clin Endocrinol Metab 23:181–192
42. Speiser PW, White PC (2003) Congenital adrenal hyperplasia. N Engl J Med 349:776–788
43. Oakes MB, Eyvazzadeh AD, Quint E, Smith YR (2008) Complete androgen insensitivity syndrome—a review. J Pediatr Adolesc Gynecol 21:305–310
44. Fechner A, Fong S, McGovern P (2008) A review of Kallmann syndrome: genetics, pathophysiology, and clinical management. Obstet Gynecol Surv 63:189–194
45. Paduch DA, Fine RG, Bolyakov A, Kiper J (2008) New concepts in Klinefelter syndrome. Curr Opin Urol 18:621–627
46. Visootsak J, Graham JM Jr (2006) Klinefelter syndrome and other sex chromosomal aneuploidies. Orphanet J Rare Dis 1:42
47. Ross MT, Grafham DV, Coffey AJ, Scherer S, McLay K, Muzny D, Platzer M, Howell GR, Burrows C, Bird CP *et al* (2005) The DNA sequence of the human X chromosome. Nature 434:325–337
48. Colborn T, vom Saal FS, Soto AM (1993) Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect 101:378–384

49. Mosher WD, Martinez GM, Chandra A, Abma JC, Willson SJ (2004) Use of contraception and use of family planning services in the United States: 1982-2002. Adv Data (350):1–36
50. Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT (2002) Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance. Environ Sci Technol 36:1202–1211
51. Jobling S, Williams R, Johnson A, Taylor A, Gross-Sorokin M, Nolan M, Tyler CR, van Aerle R, Santos E, Brighty G (2006) Predicted exposures to steroid estrogens in U.K. rivers correlate with widespread sexual disruption in wild fish populations. Environ Health Perspect 114(Suppl 1):32–39
52. Herbst AL, Ulfelder H, Poskanzer DC (1971) Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women. N Engl J Med 284: 878–881
53. Giusti RM, Iwamoto K, Hatch EE (1995) Diethylstilbestrol revisited: a review of the long-term health effects. Ann Intern Med 122:778–788
54. Klip H, Verloop J, van Gool JD, Koster ME, Burger CW, van Leeuwen FE (2002) Hypospadias in sons of women exposed to diethylstilbestrol *in utero*: a cohort study. Lancet 359: 1102–1107
55. Brouwers MM, Feitz WF, Roelofs LA, Kiemeney LA, de Gier RP, Roeleveld N (2006) Hypospadias: a transgenerational effect of diethylstilbestrol? Hum Reprod 21:666–669
56. Titus-Ernstoff L, Troisi R, Hatch EE, Palmer JR, Hyer M, Kaufman R, Adam E, Noller K, Hoover RN (2009) Birth defects in the sons and daughters of women who were exposed *in utero* to diethylstilbestrol (DES). Int J Androl 33:377–384
57. Newbold RR, Tyrey S, Haney AF, McLachlan JA (1983) Developmentally arrested oviduct: a structural and functional defect in mice following prenatal exposure to diethylstilbestrol. Teratology 27:417–426
58. Li S, Washburn KA, Moore R, Uno T, Teng C, Newbold RR, McLachlan JA, Negishi M (1997) Developmental exposure to diethylstilbestrol elicits demethylation of estrogen-responsive lactoferrin gene in mouse uterus. Cancer Res 57:4356–4359
59. Zheng X, Hendry WJ 3rd (1997) Neonatal diethylstilbestrol treatment alters the estrogen-regulated expression of both cell proliferation and apoptosis-related proto-oncogenes (c-jun, c-fos, c-myc, bax, bcl-2, and bcl-x) in the hamster uterus. Cell Growth Differ 8:425–434
60. Nef S, Shipman T, Parada LF (2000) A molecular basis for estrogen-induced cryptorchidism. Dev Biol 224:354–361
61. Cederroth CR, Schaad O, Descombes P, Chambon P, Vassalli JD, Nef S (2007) Estrogen receptor α is a major contributor to estrogen-mediated fetal testis dysgenesis and cryptorchidism. Endocrinology 148:5507–5519
62. Guyot R, Odet F, Leduque P, Forest MG, Le Magueresse-Battistoni B (2004) Diethylstilbestrol inhibits the expression of the steroidogenic acute regulatory protein in mouse fetal testis. Mol Cell Endocrinol 220:67–75
63. Haavisto T, Nurmela K, Pohjanvirta R, Huuskonen H, El-Gehani F, Paranko J (2001) Prenatal testosterone and luteinizing hormone levels in male rats exposed during pregnancy to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and diethylstilbestrol. Mol Cell Endocrinol 178:169–179
64. Sharpe RM, Irvine DS (2004) How strong is the evidence of a link between environmental chemicals and adverse effects on human reproductive health? Br Med J 328:447–451
65. Hoei-Hansen CE, Holm M, Rajpert-De Meyts E, Skakkebaek NE (2003) Histological evidence of testicular dysgenesis in contralateral biopsies from 218 patients with testicular germ cell cancer. J Pathol 200:370–374
66. Colvin JS, Green RP, Schmahl J, Capel B, Ornitz DM (2001) Male-to-female sex reversal in mice lacking fibroblast growth factor 9. Cell 104:875–889
67. Jeffs B, Meeks JJ, Ito M, Martinson FA, Matzuk MM, Jameson JL, Russell LD (2001) Blockage of the rete testis and efferent ductules by ectopic Sertoli and Leydig cells causes infertility in Dax1-deficient male mice. Endocrinology 142:4486–4495
68. Nef S, Parada LF (1999) Cryptorchidism in mice mutant for Insl3. Nat Genet 22:295–299

69. Thomas JA, Thomas MJ (1984) Biological effects of di-(2-ethylhexyl) phthalate and other phthalic acid esters. Crit Rev Toxicol 13:283–317
70. Blount BC, Silva MJ, Caudill SP, Needham LL, Pirkle JL, Sampson EJ, Lucier GW, Jackson RJ, Brock JW (2000) Levels of seven urinary phthalate metabolites in a human reference population. Environ Health Perspect 108:979–982
71. Loff S, Kabs F, Witt K, Sartoris J, Mandl B, Niessen KH, Waag KL (2000) Polyvinylchloride infusion lines expose infants to large amounts of toxic plasticizers. J Pediatr Surg 35: 1775–1781
72. Fisher JS (2004) Environmental anti-androgens and male reproductive health: focus on phthalates and testicular dysgenesis syndrome. Reproduction 127:305–315
73. Latini G, Del Vecchio A, Massaro M, Verrotti A, De Felice C (2006) Phthalate exposure and male infertility. Toxicology 226:90–98
74. Sharpe RM, Skakkebaek NE (2008) Testicular dysgenesis syndrome: mechanistic insights and potential new downstream effects. Fertil Steril 89:e33–e38
75. Kavlock R, Barr D, Boekelheide K, Breslin W, Breysse P, Chapin R, Gaido K, Hodgson E, Marcus M, Shea K, Williams P (2006) NTP-CERHR Expert Panel update on the reproductive and developmental toxicity of di(2-ethylhexyl) phthalate. Reprod Toxicol 22:291–399
76. NTP (1991) Final report on the reproductive toxicity of di(n-butyl)phthalate (CAS No. 84-74-2) in Sprague–Dawley rats. National Toxicology Program (NTP), National Technical Information Service (NTIS), U.S. Department of Commerce, Springfield, VA
77. Jobling S, Reynolds T, White R, Parker MG, Sumpter JP (1995) A variety of environmentally persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. Environ Health Perspect 103:582–587
78. Gray LE Jr, Ostby J, Ferrell J, Rehnberg G, Linder R, Cooper R, Goldman J, Slott V, Laskey J (1989) A dose-response analysis of methoxychlor-induced alterations of reproductive development and function in the rat. Fundam Appl Toxicol 12:92–108
79. Mylchreest E, Cattley RC, Foster PM (1998) Male reproductive tract malformations in rats following gestational and lactational exposure to di(*n*-butyl) phthalate: an antiandrogenic mechanism? Toxicol Sci 43:47–60
80. Lehmann KP, Phillips S, Sar M, Foster PM, Gaido KW (2004) Dose-dependent alterations in gene expression and testosterone synthesis in the fetal testes of male rats exposed to di(*n*-butyl) phthalate. Toxicol Sci 81:60–68
81. Mylchreest E, Sar M, Cattley RC, Foster PM (1999) Disruption of androgen-regulated male reproductive development by di(*n*-butyl) phthalate during late gestation in rats is different from flutamide. Toxicol Appl Pharmacol 156:81–95
82. Mylchreest E, Wallace DG, Cattley RC, Foster PM (2000) Dose-dependent alterations in androgen-regulated male reproductive development in rats exposed to di(*n*-butyl) phthalate during late gestation. Toxicol Sci 55:143–151
83. Shultz VD, Phillips S, Sar M, Foster PM, Gaido KW (2001) Altered gene profiles in fetal rat testes after *in utero* exposure to di(*n*-butyl) phthalate. Toxicol Sci 64:233–242
84. Mylchreest E, Sar M, Wallace DG, Foster PM (2002) Fetal testosterone insufficiency and abnormal proliferation of Leydig cells and gonocytes in rats exposed to di(*n*-butyl) phthalate. Reprod Toxicol 16:19–28
85. Barlow NJ, Phillips SL, Wallace DG, Sar M, Gaido KW, Foster PM (2003) Quantitative changes in gene expression in fetal rat testes following exposure to di(*n*-butyl) phthalate. Toxicol Sci 73:431–441
86. David RM (2006) Proposed mode of action for *in utero* effects of some phthalate esters on the developing male reproductive tract. Toxicol Pathol 34:209–219
87. Hallmark N, Walker M, McKinnell C, Mahood IK, Scott H, Bayne R, Coutts S, Anderson RA, Greig I, Morris K, Sharpe RM (2007) Effects of monobutyl and di(*n*-butyl) phthalate *in vitro* on steroidogenesis and Leydig cell aggregation in fetal testis explants from the rat: comparison with effects *in vivo* in the fetal rat and neonatal marmoset and *in vitro* in the human. Environ Health Perspect 115:390–396

88. Chauvigne F, Menuet A, Lesne L, Chagnon M, Chevrier C, Regnier J, Angerer J, Jegou B (2009) Time- and dose-related effects of di-(2-ethylhexyl) phthalate and its main metabolites on the function of the rat fetal testis *in vitro*. Environ Health Perspect 117:515–521
89. Li H, Kim KH (2003) Effects of mono-(2-ethylhexyl) phthalate on fetal and neonatal rat testis organ cultures. Biol Reprod 69:1964–1972
90. Stroheker T, Regnier JF, Lassurguere J, Chagnon MC (2006) Effect of *in utero* exposure to di-(2-ethylhexyl)phthalate: distribution in the rat fetus and testosterone production by rat fetal testis in culture. Food Chem Toxicol 44:2064–2069
91. Traggiai C, Stanhope R (2003) Disorders of pubertal development. Best Pract Res Clin Obstet Gynaecol 17:41–56
92. Brook CG, Jacobs HS, Stanhope R, Adams J, Hindmarsh P (1987) Pulsatility of reproductive hormones: applications to the understanding of puberty and to the treatment of infertility. Baillieres Clin Endocrinol Metab 1:23–41
93. Viswanathan V, Eugster EA (2009) Etiology and treatment of hypogonadism in adolescents. Endocrinol Metab Clin North Am 38:719–738
94. Furuya S, Kumamoto Y, Sugiyama S (1978) Fine structure and development of Sertoli junctions in human testis. Arch Androl 1:211–219
95. Wong CH, Cheng CY (2005) The blood-testis barrier: its biology, regulation, and physiological role in spermatogenesis. Curr Top Dev Biol 71:263–296
96. Moroi S, Saitou M, Fujimoto K, Sakakibara A, Furuse M, Yoshida O, Tsukita S (1998) Occludin is concentrated at tight junctions of mouse/rat but not human/guinea pig Sertoli cells in testes. Am J Physiol 274:C1708–C1717
97. Gow A, Southwood CM, Li JS, Pariali M, Riordan GP, Brodie SE, Danias J, Bronstein JM, Kachar B, Lazzarini RA (1999) CNS myelin and sertoli cell tight junction strands are absent in Osp/claudin-11 null mice. Cell 99:649–659
98. Gliki G, Ebnet K, Aurrand-Lions M, Imhof BA, Adams RH (2004) Spermatid differentiation requires the assembly of a cell polarity complex downstream of junctional adhesion molecule-C. Nature 431:320–324
99. Lee NP, Mruk D, Lee WM, Cheng CY (2003) Is the cadherin/catenin complex a functional unit of cell-cell actin-based adherens junctions in the rat testis? Biol Reprod 68: 489–508
100. Mueller S, Rosenquist TA, Takai Y, Bronson RA, Wimmer E (2003) Loss of nectin-2 at Sertoli-spermatid junctions leads to male infertility and correlates with severe spermatozoan head and midpiece malformation, impaired binding to the zona pellucida, and oocyte penetration. Biol Reprod 69:1330–1340
101. Russell L (1977) Movement of spermatocytes from the basal to the adluminal compartment of the rat testis. Am J Anat 148:313–328
102. Yu WJ, Lee BJ, Nam SY, Ahn B, Hong JT, Do JC, Kim YC, Lee YS, Yun YW (2004) Reproductive disorders in pubertal and adult phase of the male rats exposed to vinclozolin during puberty. J Vet Med Sci 66:847–853
103. Blystone CR, Lambright CS, Cardon MC, Furr J, Rider CV, Hartig PC, Wilson VS, Gray LE Jr (2009) Cumulative and antagonistic effects of a mixture of the antiandrogens vinclozolin and iprodione in the pubertal male rat. Toxicol Sci 111:179–188
104. Russell LD, Ettlin RA, Sinha Hikim AP, Clegg ED (1990) Histological and histopathological evaluation of the testis. Cache River Press, Clearwater, FL
105. Lee TL, Pang AL, Rennert OM, Chan WY (2009) Genomic landscape of developing male germ cells. Birth Defects Res C Embryo Today 87:43–63
106. Dadoune JP, Siffroi JP, Alfonsi MF (2004) Transcription in haploid male germ cells. Int Rev Cytol 237:1–56
107. Ostermeier GC, Dix DJ, Miller D, Khatri P, Krawetz SA (2002) Spermatozoal RNA profiles of normal fertile men. Lancet 360:772–777
108. Krawetz SA (2005) Paternal contribution: new insights and future challenges. Nat Rev Genet 6:633–642

109. Miller D, Ostermeier GC, Krawetz SA (2005) The controversy, potential and roles of spermatozoal RNA. Trends Mol Med 11:156–163
110. Hinton BT, Palladino MA (1995) Epididymal epithelium: its contribution to the formation of a luminal fluid microenvironment. Microsc Res Tech 30:67–81
111. Boue F, Lassalle B, Duquenne C, Villaroya S, Testart J, Lefevre A, Finaz C (1992) Human sperm proteins from testicular and epididymal origin that participate in fertilization: modulation of sperm binding to zona-free hamster oocytes, using monoclonal antibodies. Mol Reprod Dev 33:470–480
112. McNeal JE (1988) Normal histology of the prostate. Am J Surg Pathol 12:619–633
113. Lee C, Keefer M, Zhao ZW, Kroes R, Berg L, Liu XX, Sensibar J (1989) Demonstration of the role of prostate-specific antigen in semen liquefaction by two-dimensional electrophoresis. J Androl 10:432–438
114. Boekelheide K, Fleming SL, Allio T, Embree-Ku ME, Hall SJ, Johnson KJ, Kwon EJ, Patel SR, Rasoulpour RJ, Schoenfeld HA, Thompson S (2003) 2,5-Hexanedione-induced testicular injury. Annu Rev Pharmacol Toxicol 43:125–147
115. Bjorge C, Wiger R, Holme JA, Brunborg G, Andersen R, Dybing E, Soderlund EJ (1995) *In vitro* toxicity of 1,2-dibromo-3-chloropropane (DBCP) in different testicular cell types from rats. Reprod Toxicol 9:461–473
116. Slutsky M, Levin JL, Levy BS (1999) Azoospermia and oligospermia among a large cohort of DBCP applicators in 12 countries. Int J Occup Environ Health 5:116–122
117. Amann RP, Berndtson WE (1986) Assessment of procedures for screening agents for effects on male reproduction: effects of dibromochloropropane (DBCP) on the rat. Fundam Appl Toxicol 7:244–255
118. Pearson PG, Soderlund EJ, Dybing E, Nelson SD (1990) Metabolic activation of 1,2-dibromo-3-chloropropane: evidence for the formation of reactive episulfonium ion intermediates. Biochemistry 29:4971–4981
119. Taylor MF, de Boer-Brouwer M, Woolveridge I, Teerds KJ, Morris ID (1999) Leydig cell apoptosis after the administration of ethane dimethanesulfonate to the adult male rat is a Fas-mediated process. Endocrinology 140:3797–3804
120. Bakalska M, Atanassova N, Angelova P, Koeva I, Nikolov B, Davidoff M (2001) Degeneration and restoration of spermatogenesis in relation to the changes in Leydig cell population following ethane dimethanesulfonate treatment in adult rats. Endocr Regul 35:209–215
121. Moffit JS, Bryant BH, Hall SJ, Boekelheide K (2007) Dose-dependent effects of sertoli cell toxicants 2,5-hexanedione, carbendazim, and mono-(2-ethylhexyl) phthalate in adult rat testis. Toxicol Pathol 35:719–727
122. Yu G, Guo Q, Xie L, Liu Y, Wang X (2009) Effects of subchronic exposure to carbendazim on spermatogenesis and fertility in male rats. Toxicol Ind Health 25:41–47
123. Noorafshan A, Karbalay-Doust S, Ardekani FM (2005) High doses of nandrolone decanoate reduce volume of testis and length of seminiferous tubules in rats. APMIS 113: 122–125
124. O'Sullivan AJ, Kennedy MC, Casey JH, Day RO, Corrigan B, Wodak AD (2000) Anabolic-androgenic steroids: medical assessment of present, past and potential users. Med J Aust 173:323–327
125. Takahashi M, Tatsugi Y, Kohno T (2004) Endocrinological and pathological effects of anabolic-androgenic steroid in male rats. Endocr J 51:425–434
126. Rasoulpour RJ, Schoenfeld HA, Gray DA, Boekelheide K (2003) Expression of a K48R mutant ubiquitin protects mouse testis from cryptorchid injury and aging. Am J Pathol 163: 2595–2603
127. Richburg JH, Johnson KJ, Schoenfeld HA, Meistrich ML, Dix DJ (2002) Defining the cellular and molecular mechanisms of toxicant action in the testis. Toxicol Lett 135:167–183
128. d'Ancona FC, Debruyne FM (2005) Endocrine approaches in the therapy of prostate carcinoma. Hum Reprod Update 11:309–317

129. Schoenfeld HA, Hall SJ, Boekelheide K (2001) Continuously proliferative stem germ cells partially repopulate the aged, atrophic rat testis after gonadotropin-releasing hormone agonist therapy. Biol Reprod 64:1273–1282
130. Wang H, Zhou Z, Xu M, Li J, Xiao J, Xu ZY, Sha J (2004) A spermatogenesis-related gene expression profile in human spermatozoa and its potential clinical applications. J Mol Med 82:317–324
131. Aoki VW, Liu L, Carrell DT (2006) A novel mechanism of protamine expression deregulation highlighted by abnormal protamine transcript retention in infertile human males with sperm protamine deficiency. Mol Hum Reprod 12:41–50
132. Cho C, Willis WD, Goulding EH, Jung-Ha H, Choi YC, Hecht NB, Eddy EM (2001) Haploinsufficiency of protamine-1 or -2 causes infertility in mice. Nat Genet 28:82–86
133. Steger K, Wilhelm J, Konrad L, Stalf T, Greb R, Diemer T, Kliesch S, Bergmann M, Weidner W (2008) Both protamine-1 to protamine-2 mRNA ratio and Bcl2 mRNA content in testicular spermatids and ejaculated spermatozoa discriminate between fertile and infertile men. Hum Reprod 23:11–16
134. Klinefelter GR (2008) Saga of a sperm fertility biomarker. Anim Reprod Sci 105:90–103
135. Paulsen M, Ferguson-Smith AC (2001) DNA methylation in genomic imprinting, development, and disease. J Pathol 195:97–110
136. Li E, Bestor TH, Jaenisch R (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 69:915–926
137. Okano M, Bell DW, Haber DA, Li E (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for *de novo* methylation and mammalian development. Cell 99: 247–257
138. Niemitz EL, Feinberg AP (2004) Epigenetics and assisted reproductive technology: a call for investigation. Am J Hum Genet 74:599–609
139. Weber M, Davies JJ, Wittig D, Oakeley EJ, Haase M, Lam WL, Schubeler D (2005) Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. Nat Genet 37:853–862
140. Houshdaran S, Cortessis VK, Siegmund K, Yang A, Laird PW, Sokol RZ (2007) Widespread epigenetic abnormalities suggest a broad DNA methylation erasure defect in abnormal human sperm. PLoS One 2:e1289
141. Pathak S, Kedia-Mokashi N, Saxena M, D'Souza R, Maitra A, Parte P, Gill-Sharma M, Balasinor N (2009) Effect of tamoxifen treatment on global and insulin-like growth factor 2-H19 locus-specific DNA methylation in rat spermatozoa and its association with embryo loss. Fertil Steril 91:2253–2263
142. Plas E, Berger P, Hermann M, Pfluger H (2000) Effects of aging on male fertility? Exp Gerontol 35:543–551
143. Hermann M, Berger P (2001) Hormonal changes in aging men: a therapeutic indication? Exp Gerontol 36:1075–1082
144. Tsutsumi R, Webster NJ (2009) GnRH pulsatility, the pituitary response and reproductive dysfunction. Endocr J 56:729–737
145. Zirkin BR, Santulli R, Strandberg JD, Wright WW, Ewing LL (1993) Testicular steroidogenesis in the aging brown Norway rat. J Androl 14:118–123
146. Wang C, Sinha Hikim AP, Lue YH, Leung A, Baravarian S, Swerdloff RS (1999) Reproductive aging in the Brown Norway rat is characterized by accelerated germ cell apoptosis and is not altered by luteinizing hormone replacement. J Androl 20:509–518
147. Chen H, Hardy MP, Huhtaniemi I, Zirkin BR (1994) Age-related decreased Leydig cell testosterone production in the brown Norway rat. J Androl 15:551–557
148. Kim IS, Ariyaratne HB, Mendis-Handagama SM (2002) Changes in the testis interstitium of Brown Norway rats with aging and effects of luteinizing and thyroid hormones on the aged testes in enhancing the steroidogenic potential. Biol Reprod 66:1359–1366
149. Birnbaum LS (1991) Pharmacokinetic basis of age-related changes in sensitivity to toxicants. Annu Rev Pharmacol Toxicol 31:101–128

150. Jackson JA, Birnbaum LS, Diliberto JJ (1998) Effects of age, sex, and pharmacologic agents on the biliary elimination of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in F344 rats. Drug Metab Dispos 26:714–719
151. Bliss CI (1939) The toxicity of poisons applied jointly. Ann Appl Biol 27:585–615
152. Parvez S, Venkataraman C, Mukherji S (2009) Nature and prevalence of non-additive toxic effects in industrially relevant mixtures of organic chemicals. Chemosphere 75:1429–1439
153. Rider CV, Wilson VS, Howdeshell KL, Hotchkiss AK, Furr JR, Lambright CR, Gray LE Jr (2009) Cumulative effects of *in utero* administration of mixtures of "antiandrogens" on male rat reproductive development. Toxicol Pathol 37:100–113
154. Howdeshell KL, Furr J, Lambright CR, Rider CV, Wilson VS, Gray LE Jr (2007) Cumulative effects of dibutyl phthalate and diethylhexyl phthalate on male rat reproductive tract development: altered fetal steroid hormones and genes. Toxicol Sci 99:190–202
155. Rider CV, Furr J, Wilson VS, Gray LE Jr (2008) A mixture of seven antiandrogens induces reproductive malformations in rats. Int J Androl 31:249–262
156. Andric SA, Kostic TS, Stojilkovic SS, Kovacevic RZ (2000) Inhibition of rat testicular androgenesis by a polychlorinated biphenyl mixture aroclor 1248. Biol Reprod 62:1882–1888
157. Markelewicz RJ Jr, Hall SJ, Boekelheide K (2004) 2,5-Hexanedione and carbendazim coexposure synergistically disrupts rat spermatogenesis despite opposing molecular effects on microtubules. Toxicol Sci 80:92–100
158. Calabrese EJ (2005) Paradigm lost, paradigm found: the re-emergence of hormesis as a fundamental dose response model in the toxicological sciences. Environ Pollut 138:379–411
159. NTP (2001) NTP report of the endocrine disruptors low-dose peer review. National Toxicology Program (NTP), National Institute of Environmental Health Sciences, Research Triangle Park, NC
160. Razzaghi M, Loomis P (2001) The concept of hormesis in developmental toxicology. Hum Ecologic Risk Assess 7:933–942
161. Mushak P (2007) Hormesis and its place in nonmonotonic dose-response relationships: some scientific reality checks. Environ Health Perspect 115:500–506
162. Falls JG, Pulford DJ, Wylie AA, Jirtle RL (1999) Genomic imprinting: implications for human disease. Am J Pathol 154:635–647
163. Weaver JR, Susiarjo M, Bartolomei MS (2009) Imprinting and epigenetic changes in the early embryo. Mamm Genome 20:532–543
164. Biliya S, Bulla LA Jr (2010) Genomic imprinting: the influence of differential methylation in the two sexes. Exp Biol Med 235:139–147
165. Oswald J, Engemann S, Lane N, Mayer W, Olek A, Fundele R, Dean W, Reik W, Walter J (2000) Active demethylation of the paternal genome in the mouse zygote. Curr Biol 10:475–478
166. Mayer W, Niveleau A, Walter J, Fundele R, Haaf T (2000) Demethylation of the zygotic paternal genome. Nature 403:501–502
167. Kierszenbaum AL (2002) Genomic imprinting and epigenetic reprogramming: unearthing the garden of forking paths. Mol Reprod Dev 63:269–272
168. Reik W, Dean W, Walter J (2001) Epigenetic reprogramming in mammalian development. Science 293:1089–1093
169. Trasler JM (2009) Epigenetics in spermatogenesis. Mol Cell Endocrinol 306:33–36
170. Oakes CC, La Salle S, Smiraglia DJ, Robaire B, Trasler JM (2007) Developmental acquisition of genome-wide DNA methylation occurs prior to meiosis in male germ cells. Dev Biol 307:368–379
171. Richards EJ (2006) Inherited epigenetic variation—revisiting soft inheritance. Nat Rev Genet 7:395–401
172. Anway MD, Cupp AS, Uzumcu M, Skinner MK (2005) Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 308:1466–1469
173. Skinner MK (2008) What is an epigenetic transgenerational phenotype? F3 or F2. Reprod Toxicol 25:2–6

174. Chang HS, Anway MD, Rekow SS, Skinner MK (2006) Transgenerational epigenetic imprinting of the male germline by endocrine disruptor exposure during gonadal sex determination. Endocrinology 147:5524–5541
175. Anway MD, Leathers C, Skinner MK (2006) Endocrine disruptor vinclozolin induced epigenetic transgenerational adult-onset disease. Endocrinology 147:5515–5523
176. Schneider S, Kaufmann W, Buesen R, van Ravenzwaay B (2008) Vinclozolin—the lack of a transgenerational effect after oral maternal exposure during organogenesis. Reprod Toxicol 25:352–360
177. Furr J, Gray LE (2009) Vinclozolin (V) treatment induces reproductive malformation and infertility in F1 male rats when administered during sexual but not gonadal differentiation. The effects are not transmitted to the subsequent generations (abstract No. 1441). Toxicologist 108(1):298; Society of Toxicology, Baltimore, MD, USA
178. McGlynn KA, Devesa SS, Sigurdson AJ, Brown LM, Tsao L, Tarone RE (2003) Trends in the incidence of testicular germ cell tumors in the United States. Cancer 97:63–70
179. Shah MN, Devesa SS, Zhu K, McGlynn KA (2007) Trends in testicular germ cell tumours by ethnic group in the United States. Int J Androl 30:206–213; discussion 213–204
180. Lacerda HM, Akre O, Merletti F, Richiardi L (2009) Time trends in the incidence of testicular cancer in childhood and young adulthood. Cancer Epidemiol Biomarkers Prev 18:2042–2045
181. Rajpert-De Meyts E (2006) Developmental model for the pathogenesis of testicular carcinoma *in situ*: genetic and environmental aspects. Hum Reprod Update 12:303–323
182. Ahlgren M, Wohlfahrt J, Olsen LW, Sorensen TI, Melbye M (2007) Birth weight and risk of cancer. Cancer 110:412–419
183. Michos A, Xue F, Michels KB (2007) Birth weight and the risk of testicular cancer: a meta-analysis. Int J Cancer 121:1123–1131
184. Neale RE, Carriere P, Murphy MF, Baade PD (2008) Testicular cancer in twins: a meta-analysis. Br J Cancer 98:171–173
185. Swerdlow AJ, De Stavola BL, Swanwick MA, Mangtani P, Maconochie NE (1999) Risk factors for testicular cancer: a case-control study in twins. Br J Cancer 80:1098–1102
186. Hoei-Hansen CE, Olesen IA, Jorgensen N, Carlsen E, Holm M, Almstrup K, Leffers H, Rajpert-De Meyts E (2007) Current approaches for detection of carcinoma *in situ* testis. Int J Androl 30:398–404; discussion 404–395
187. Skakkebaek NE, Rajpert-De Meyts E, Jorgensen N, Main KM, Leffers H, Andersson AM, Juul A, Jensen TK, Toppari J (2007) Testicular cancer trends as 'whistle blowers' of testicular developmental problems in populations. Int J Androl 30:198–204; discussion 204–195
188. Rajpert-De Meyts E, Bartkova J, Samson M, Hoei-Hansen CE, Frydelund-Larsen L, Bartek J, Skakkebaek NE (2003) The emerging phenotype of the testicular carcinoma *in situ* germ cell. APMIS 111:267–278; discussion 278–269
189. Rajpert-de Meyts E, Hoei-Hansen CE (2007) From gonocytes to testicular cancer: the role of impaired gonadal development. Ann NY Acad Sci 1120:168–180
190. Barlow NJ, Foster PM (2003) Pathogenesis of male reproductive tract lesions from gestation through adulthood following *in utero* exposure to di(*n*-butyl) phthalate. Toxicol Pathol 31: 397–410
191. Ferrara D, Hallmark N, Scott H, Brown R, McKinnell C, Mahood IK, Sharpe RM (2006) Acute and long-term effects of *in utero* exposure of rats to di(*n*-butyl) phthalate on testicular germ cell development and proliferation. Endocrinology 147:5352–5362
192. Olesen IA, Sonne SB, Hoei-Hansen CE, Rajpert-DeMeyts E, Skakkebaek NE (2007) Environment, testicular dysgenesis and carcinoma *in situ* testis. Best Pract Res Clin Endocrinol Metab 21:462–478
193. Krentz AD, Murphy MW, Kim S, Cook MS, Capel B, Zhu R, Matin A, Sarver AL, Parker KL, Griswold MD, Looijenga LH, Bardwell VJ, Zarkower D (2009) The DM domain protein DMRT1 is a dose-sensitive regulator of fetal germ cell proliferation and pluripotency. Proc Natl Acad Sci USA 106:22323–22328

194. Jiang LI, Nadeau JH (2001) 129/Sv mice—a model system for studying germ cell biology and testicular cancer. Mamm Genome 12:89–94
195. Youngren KK, Coveney D, Peng XN, Bhattacharya C, Schmidt LS, Nickerson ML, Lamb BT, Deng JM, Behringer RR, Capel B, Rubin EM, Nadeau JH, Matin A (2005) The Ter mutation in the dead end gene causes germ cell loss and testicular germ cell tumours. Nature 435:360–364
196. Meng X, de Rooij DG, Westerdahl K, Saarma M, Sariola H (2001) Promotion of seminomatous tumors by targeted overexpression of glial cell line-derived neurotrophic factor in mouse testis. Cancer Res 61:3267–3271
197. Donehower LA (1996) The p53-deficient mouse: a model for basic and applied cancer studies. Semin Cancer Biol 7:269–278
198. Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA Jr, Butel JS, Bradley A (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature 356:215–221
199. Harvey M, McArthur MJ, Montgomery CA Jr, Butel JS, Bradley A, Donehower LA (1993) Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice. Nat Genet 5: 225–229
200. Harvey M, McArthur MJ, Montgomery CA Jr, Bradley A, Donehower LA (1993) Genetic background alters the spectrum of tumors that develop in p53-deficient mice. FASEB J 7:938–943
201. Xia T, Blackburn WR, Gardner WA Jr (1990) Fetal prostate growth and development. Pediatr Pathol 10:527–537
202. Cunha GR (1994) Role of mesenchymal-epithelial interactions in normal and abnormal development of the mammary gland and prostate. Cancer 74:1030–1044
203. Hayward SW, Cunha GR, Dahiya R (1996) Normal development and carcinogenesis of the prostate. A unifying hypothesis. Ann NY Acad Sci 784:50–62
204. Cunha GR, Alarid ET, Turner T, Donjacour AA, Boutin EL, Foster BA (1992) Normal and abnormal development of the male urogenital tract. Role of androgens, mesenchymal-epithelial interactions, and growth factors. J Androl 13:465–475
205. Ekbom A (1998) Growing evidence that several human cancers may originate *in utero*. Semin Cancer Biol 8:237–244
206. Ellem SJ, Risbridger GP (2009) The dual, opposing roles of estrogen in the prostate. Ann NY Acad Sci 1155:174–186
207. Adams JY, Leav I, Lau KM, Ho SM, Pflueger SM (2002) Expression of estrogen receptor beta in the fetal, neonatal, and prepubertal human prostate. Prostate 52:69–81
208. Aksglaede L, Juul A, Leffers H, Skakkebaek NE, Andersson AM (2006) The sensitivity of the child to sex steroids: possible impact of exogenous estrogens. Hum Reprod Update 12:341–349
209. Bosland MC (2005) The role of estrogens in prostate carcinogenesis: a rationale for chemoprevention. Rev Urol 7(Suppl 3):S4–S10
210. Sugimura Y, Cunha GR, Yonemura CU, Kawamura J (1988) Temporal and spatial factors in diethylstilbestrol-induced squamous metaplasia of the developing human prostate. Hum Pathol 19:133–139
211. Yonemura CY, Cunha GR, Sugimura Y, Mee SL (1995) Temporal and spatial factors in diethylstilbestrol-induced squamous metaplasia in the developing human prostate. II. Persistent changes after removal of diethylstilbestrol. Acta Anat 153:1–11
212. Bosland MC, Ford H, Horton L (1995) Induction at high incidence of ductal prostate adenocarcinomas in NBL/Cr and Sprague-Dawley Hsd:SD rats treated with a combination of testosterone and estradiol-17β or diethylstilbestrol. Carcinogenesis 16:1311–1317
213. Prins GS, Tang WY, Belmonte J, Ho SM (2008) Perinatal exposure to oestradiol and bisphenol A alters the prostate epigenome and increases susceptibility to carcinogenesis. Basic Clin Pharmacol Toxicol 102:134–138
214. Prins GS, Birch L, Tang WY, Ho SM (2007) Developmental estrogen exposures predispose to prostate carcinogenesis with aging. Reprod Toxicol 23:374–382

Interspecies Uncertainty in Molecular Responses and Toxicity of Mixtures

Moiz M. Mumtaz and Hana R. Pohl

Abstract Most of the experimental toxicity testing data for chemicals are generated through the use of laboratory animals, namely, rodents such as rats and mice or other species. Interspecies extrapolation is needed to nullify the differences between species so as to use such data for human health/risk assessment. Thus, understanding of interspecies differences is important in extrapolating the laboratory results to humans and conducting human risk assessments based on current credible scientific knowledge. Major causes of interspecies differences in anatomy and physiology, toxicokinetics, injury repair, molecular receptors, and signal transduction pathways responsible for variations in responses to toxic chemicals are outlined. In the risk assessment process, uncertainty associated with data gaps in our knowledge is reflected by application of uncertainty factors for interspecies differences. Refinement of the risk assessment methods is the ultimate goal as we strive to realistically evaluate the impact of toxic chemicals on human populations. Using specific examples from current risk assessment practice, this chapter illustrates the integration of interspecies differences in evaluation of individual chemicals and chemical mixtures.

Keywords Chemical interactions · Complex mixtures · Cytochrome P450-dependent monooxygenases · Interspecies variation · Metabolic variation · Molecular receptor variation · 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) · Minimal risk level · Human equivalent concentration · Signal transduction variation ·

Note: The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Agency for Toxic Substances and Disease Registry (ATSDR)

M.M. Mumtaz (✉) and H.R. Pohl
Division of Toxicology and Human Health Sciences, Agency of Toxic Substances and Disease Registry (ATSDR)
Centers for Disease Control and Prevention (CDC), U.S. Department of Health and Human Services, Atlanta, GA 30333, USA
e-mail: mgm4@cdc.gov; hpohl@cdc.gov

A. Luch (ed.), *Molecular, Clinical and Environmental Toxicology*,
Experientia Supplementum 101, DOI 10.1007/978-3-7643-8340-4_12,

Risk assessment · Threshold limit values · Tissue repair variation · Toxic equivalent · Weight of evidence

Introduction

For an evaluation of the health effects of exposure to chemicals in human population, evidence of adverse effects should be obtained from human studies, but rarely relevant toxicity data are available in humans. Although many epidemiological studies are available that identify adverse effects in human populations, these studies often suffer from a number of confounding factors. Clinical studies are scarce and refer mostly to sporadic cases of acute poisoning where the information about actual exposure is not available. Thus, there exist huge data gaps in our knowledge regarding human toxicity and mode of action of the approximately 80,000 chemicals used in commerce today. However, experimental toxicity testing data are often available in laboratory animals. Therefore, risk assessors rely heavily on animal studies to assess the potential health risks to humans. Interspecies extrapolation is an important aspect of the toxicity assessment of such chemicals. Before considering the use of such data across species, a clear understanding of a chemical's mode of action is needed.

The basic origin of species differences in response to an exposure(s) occurs at toxicokinetic or toxicodynamic levels as the chemical moves through a living system or body. Thus, a complex network of common pathways that superimpose during its disposition (absorption, distribution, metabolism, and excretion), interaction with molecular receptors, and signal transduction play a critical role in species variation and responses. The overall tissue injury and repair, after a toxic assault, are dependent on the regenerative or adaptive capabilities of individual species. To address this issue, the classical approach to risk assessment of chemicals uses uncertainty factors to extrapolate results from one species to the other. In this chapter, we present some salient examples of a few specific fundamental interspecies differences and their use in current risk assessment practices.

Interspecies Variation

Anatomical and Physiological Variation

The rationale for extrapolation or "scaling" across species is founded in the commonality of anatomic characteristics and the universality of physiologic functions and biochemical reactions among species. A study of variation in features of anatomy and physiology as a function of overall body size, allometry, reveals several biologic and physiologic parameters that vary predictably with body

weight (W) or surface area across animal species [1, 2]. The pharmacokinetic properties of chemicals also show consistent correlation with W because they are controlled by these physiologic parameters. This relationship could be expressed by an equation:

$$Y = aW^b,$$

where Y is a biological variable parameter considered whose value is dependent on W, the body weight or surface area, and "a" is the proportionality constant and "b" is the slope of the straight line defining the relationship between the value of the parameter and the weight of the animal. This equation can also be written in logarithmic terms:

$$\log Y = \log a + b \log W,$$

where "a" is equal to Y when W equals 1. If W is expressed in kilograms, "a" refers to a body weight of 1 kg; "b" is the slope of a straight line defining the relationship between the value of the parameter and the weight of the animal. The proportionality between Y and W is expressed by b. The closer the value of b is to 1, the more closely Y correlates directly with W. A plot on log–log scale of such relationships produces straight lines. The slopes for the various processes might change, but the values for humans correlate linearly with other animals.

Studies have shown that several biologic processes vary over species, according to this formula [1]. As a general rule, organ volumes and capacities tend to increase in direct proportion to body weight ($b = 1$), while ventilation rates, blood flows, and metabolic rates tend to relate well with a fractional power of body weight ($b < 1$) [3, 4]. Values of b determined for representative physiological and pharmacokinetic processes are renal clearance (0.75), volume of distribution (1.00) and biological half-life (0.25), total body volume (1.02), mass of mammalian heart (0.98), cardiac output (0.75), minute volume (0.75), and oxygen consumption (0.75). When the constant b equals two-thirds, the parameter Y correlates with the surface area.

Metabolic Variations

A major source of variation among species in the pharmacokinetic phase occurs during the metabolism of chemicals leading to qualitative and quantitative differences in the major and minor metabolites formed. These differences can be highlighted through the following two examples.

Ethylbenzene

A review of the metabolism of ethylbenzene studied in multiple species reveals that the initial step in the metabolic pathway is hydroxylation of the side chain of ethylbenzene to produce 1-phenylethanol. 1-Phenylethanol is conjugated to glucuronide, which then is either excreted or converted to other metabolites. Oxidation of 1-phenylethanol yields acetophenone, which is excreted in the urine as a minor metabolite or further transformed. Continued oxidation of the side chain leads to the sequential formation of 2-hydroxyacetophenone, 1-phenyl-1,2-ethanediol, mandelic acid, and phenylglyoxylic acid. These acids are the major metabolites of ethylbenzene in humans exposed by inhalation [5]. Minor pathways (e.g., ring hydroxylation) include glucuronide and sulfate conjugation with hydroxylated derivatives to form glucuronides and sulfates that are excreted in the urine. In rats exposed by inhalation or orally to ethylbenzene, the major metabolites are identified as hippuric and benzoic acids, 1-phenylethanol, and mandelic acid [6–8]. In rabbits, the most important metabolite is hippuric acid, which is probably formed by oxidative decarboxylation of phenylglyoxylic acid [9]. Rabbits have been shown to excrete higher levels of glucuronidated metabolites than do humans or rats [10–12]. Table 1 shows percentages of the absorbed dose of ethylbenzene detected as major metabolites in different species.

Styrene

There are numerous studies that investigated the metabolism of styrene in humans and animals. There are several metabolic pathways for styrene [13–15]. The primary pathway is oxidation of the side chain by cytochrome P450-dependent monooxygenases (CYPs) to form styrene 7,8-oxide. Styrene oxide is predominantly metabolized by epoxide hydrolase to form styrene glycol; the styrene glycol is subsequently converted to mandelic acid, phenylglyoxylic acid, and hippuric acid. Styrene 7,8-oxide can also be conjugated with glutathione to ultimately form phenylhydroxylethyl mercapturic acids. A minor pathway of styrene metabolism involves the formation of phenylacetaldehyde from styrene 7,8-oxide or CYP-dependent conversion of styrene to phenylethanol and subsequent metabolism to phenylacetic acid. An alternative minor pathway involves ring oxidation resulting in the production of styrene 3,4-oxide, which is further metabolized to 4-vinylphenol. The liver is the primary site of styrene metabolism and the source of styrene oxide in the blood [16]. However, styrene is also metabolized in other tissues,

Table 1 Interspecies differences in the metabolism of ethylbenzene

	Mandelic acid (%)	Phenylglyoxylic acid (%)	Hippuric and benzoic acids (%)	1-Phenylethanol (%)	References
Humans	70	25			[5, 7]
Rats	15–23	10	38	25	[6–8]

Table 2 Interspecies differences in the metabolism of styrene

	Mandelic acid, phenylglyoxylic acid, and hippuric acid (%)	Mercapturic acids (%)	Phenylacetic acid (%)	References
Humans	95	<1		[13, 16–18]
Rats	68–72	25–35	3–5	[19]
Mice	49–59	25–35	12–22	[16, 20]

particularly the lung and nasal cavity following inhalation exposure. Qualitative and quantitative interspecies differences exist in the biotransformation of styrene. Some of the differences are highlighted in Table 2 by presenting percentages of the absorbed dose of styrene detected as major metabolites in different species.

Enzymatic Variations

The above examples demonstrated interspecies differences in metabolism of environmental chemicals in regards to metabolic pathways and major metabolites. Key enzymes involved in the phase I reactions are a series of isozymes belonging to the superfamily known as the cytochrome P450 system. These CYP enzymes play an important role in the metabolism of many environmental chemicals, catalyzing both detoxification and bioactivation reactions such as hydroxylation, epoxidation, heteroatom oxidation, and reduction. In mammalian tissues, the CYP enzymes are membrane-bound. Many CYP enzymes have been identified; different species may use different enzymes to catalyze metabolic reactions of the same chemical.

Styrene

A number of CYP isozymes have the capacity to catalyze styrene to styrene oxide. In human livers, CYP2B6 was the most active isoform; the activities of CYP1A2 and CYP2E1 were about half that of CYP2B6 [21]. CYP2E1 was the main isoform at low styrene concentrations and CYP2B6 at high styrene concentrations [22]. CYPs from human lung have a very limited ability to metabolize styrene to styrene oxide [23, 24]; the capacity was 100-fold lower than in rat lung microsomes [13]. *In vitro* studies in isolated lung cells indicated that the more pneumotoxic and genotoxic form of styrene oxide, the *R*-enantiomer, was preferentially formed in mice, and the *S*-enantiomer was formed more in rats [25]. Comparison of the most active isozymes in the styrene metabolism in different species is presented in Table 3.

A study compared the kinetic constants of CYPs from the livers of humans, mice, and rats [26]. The affinity of CYPs for styrene (based on K_m values) was similar for the three species. However, the mouse had the greatest capacity to form styrene 7,8-oxide from styrene based on the V_{max} values and relative liver and body size, and humans had the lowest capacity.

Table 3 Styrene: interspecies differences in CYP utilization

	Enzyme	References
Human liver	CYP2B6 (at high concentration) CYP2E1 (at low concentration)	[21, 22]
Rat liver	CYP2B1	[21]
Mouse liver	CYP1A1	[21]
Human lung	CYP2F1	[24]
Rat/mouse lung	CYP2F2; CYP2E1	[25]

Halomethanes

Another example of the potential difference between species is quantitative and qualitative difference in phase I metabolism of trihalomethanes. In rats, trihalomethanes are substrates of CYP isoforms CYP2E1, CYP2B1, CY2B2, and CYP1A2 [27, 28]. CYP2E1 and CPY1A2 are also expressed in human tissues; however, the relevance of metabolism by CYP2B1/2 to human health is uncertain, since these isoenzymes have not been reported in human adult or fetal tissues [29, 30]. CYP2B6 is the only active member of the CYP2B subfamily in man, although the CYP2B7 gene has also been found in the genome [31].

Tissue Repair Variations

Many factors are considered as underlying causes of observed differences in the toxicity of chemicals among animal species. During the past decade the role of tissue repair has been studied extensively in animals of various species, strain, and health conditions [32–42]. Through these studies, it has been shown that toxicity is a two-step process that includes tissue injury and tissue repair (TR) that involves a plethora of variations. For example, Mongolian gerbils were found to be highly susceptible (35-fold lower LD_{50}) to carbon tetrachloride (CCl_4)-induced hepatotoxicity when compared with Sprague–Dawley (SD) rats that was attributed to extremely sluggish TR [43]. Gerbils were also remarkably resistant to chlordecone (CD)-amplified toxicity of CCl_4 [43]. CD-amplified CCl_4 toxicity is known to be due to inhibition of CCl_4-induced increase in compensatory tissue repair [32], and since TR is minimal in gerbils, the interactive toxicity does not occur. Similar species differences have been shown between rats and mice under disease conditions [36–38]. Streptozotocin (STZ)-induced type 1 diabetic rats were found to be highly sensitive to thioacetamide (TA)-induced liver injury where even a normally nonlethal dose of TA is lethal in DB rats because of compromised TR response [38, 39]. However, STZ-induced type 1 diabetic mice were completely refractory to liver injury induced by a lethal dose of TA due to their ability to mount effective TR response [37]. A classic example of strain difference in TR is observed between F344 and SD rats exposed to 1,2-dichlorobenzene (*o*-DCB) [44]. F344 rats exhibited significantly higher liver injury following exposure to *o*-DCB as compared to

SD rats treated with the same doses. However, the mortality induced by *o*-DCB is not higher in F344 rats, since these rats are capable of mounting a much stronger TR compared to SD rats [33]. The significantly higher TR in F344 rats enables them to prevent the progression of liver injury and thereby escape *o*-DCB-induced liver failure even though they suffer tenfold higher initiation of liver injury than the SD rats [33, 34]. When reevaluating several comparative studies of DNA repair in a variety of species, the authors reported a sixfold range of relative DNA repair activity [45]. Small rodents such as shrews, mice, and rats had the lowest and humans and gorillas the highest rates. In addition, the mutagenesis and tumorigenesis proceeded about 40 times faster in mice than in humans. These findings should have implications in risk assessments using interspecies extrapolation.

Molecular Receptor Variations

Even though a majority of species differences exist in the toxicokinetics, some interspecies differences have been also observed in the toxicodynamics too.

2,3,7,8-Tetrachlorodibenzo-p-dioxin

Many chlorinated dibenzo-*p*-dioxins (CDDs), chlorinated dibenzofurans (CDFs), coplanar polychlorinated biphenyls (PCBs), and other structurally related halogenated aromatic hydrocarbons are believed to share a common mechanism of action related to similarities in their structural configuration. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic congener. Most of what is known regarding the mechanism of action of these compounds is based on research in three main areas: structure–activity relationships for receptor binding and induction of a variety of biochemical and toxicological responses, genetic studies using inbred mouse strains, and studies at the molecular level which have elucidated key events in the actions of the receptor [46]. This receptor, the aryl hydrocarbon receptor (AhR), was first identified in the cytosol of mouse liver cells [47] and, subsequently, in hepatic and extrahepatic tissues of a variety of laboratory animals, mammalian cell cultures, human organs and cell cultures, and also nonmammalian species [48]. The proposed mechanism of action for these chemicals involves ligand binding to the cytoplasmic AhR and translocation to the nucleus. The AhR nuclear translocator (ARNT) protein interacts with the liganded AhR to form a heterodimeric DNA-binding protein complex that can bind DNA and activate gene transcription that may lead to changes in gene expression [49, 50]. Results from structure-binding relationships for a series of CDD congeners using mouse hepatic cytosol showed that not all the congeners had the same affinity for the AhR; affinity was found to be determined by the chlorine substitution pattern [47, 51, 52]. Similarly, differences in the AhR may cause interspecies differences in TCDD-induced toxicity. Decreased susceptibility to TCDD-induced toxicity was reported in mice with low-affinity AhR alleles [53], and mice with AhR-null displayed a resistance to TCDD

toxicity all together [54, 55]. Genetic polymorphism in the AhR (a 38-amino acid deletion) is associated with a 1,000-fold difference in lethality between Han–Wistar and Long-Evans strains of rats [56]. In addition, mice having mutations in the AhR nuclear localization/dioxin responsive element (DRE)-binding domain and mice having a hypomorphic ARNT allele do not show classical TCDD toxicities [57, 58]. However, not all the interspecies differences in TCDD-induced toxicity can be explained by differences in AhR (see below).

Signal Transduction Variations

Most chemicals are expected to cause adverse effects by modulating the regular signaling processes in the cell. Under this hypothesis, the chemicals induce cascades of events that start with receptor binding/activation leading to gene expression, protein expression/modification, lesion development, abnormal organ function, and, finally, disease development. Therefore, gene expression changes aid to early detection of adverse effects development. Gene expression profiles can be tested in exposed and unexposed biological samples using microarray methodology [59–61]. Similarly, samples from different species can be tested and compared.

2,3,7,8-Tetrachlorodibenzo-p-dioxin

Hepatic gene expression profiles were obtained in ovariectomized Sprague–Dawley rats and C57BL/6 mice following TCDD exposure by gavage [62]. Comparative analysis showed 33 orthologous genes that were normally regulated by TCDD as well as 185 rat-specific and 225 mouse-specific responses. The authors identified the rat-specific expression responses as being linked to cellular growth and lipid metabolism. In contrast, mouse-specific responses were associated with lipid uptake/metabolism and immune responses. Thus, the species-specific toxicity induced by TCDD may be mediated also by differences in gene expression.

Application in Risk Assessment and Current Practice

Single Chemicals

Traditionally, uncertainty factors have been used in human health risk assessment to adjust for interspecies differences. A review was performed of a number of studies that compared animal *versus* human toxicity for several toxicants [63]. The authors concluded that, in lieu of chemical-specific data, an uncertainty factor (UF) of 10 was sufficient for animal to human adjustment. Although humans are considered by some investigators to be more sensitive than animals in terms of the body

burden of chemicals [63, 64], this is not always the case. With increasing knowledge regarding interspecies differences in toxicokinetics and toxicodynamics, it became important to refine the UF approach in chemical risk assessment [65]. One such approach proposed to split the interspecies factor of 10 into a subfactor of $10^{0.6}$ (4.0) for toxicokinetics and $10^{0.4}$ (2.5) for toxicodynamics [66]. The author compared pharmacokinetic parameters of various chemicals, specifically clearance and area under the plasma or tissue concentration–time curve (AUC). The major rationale behind the final decision was the fact that rodents metabolize chemicals much faster than humans and the proposed division corresponds with the approximately fourfold difference between rats (a species commonly used in laboratory testing) and humans in basic physiological parameters that determine the clearance and elimination of chemicals (e.g., cardiac output and renal and liver blood flows). The World Health Organization (WHO) also endorsed the concept [67]. Therefore, modification of the default UF of 10 is warranted when chemical-specific information is available to support such a change.

The Agency for Toxic Substances and Disease Registry (ATSDR) derives health-based guidance values called minimal risk levels (MRLs). As of 2010, 392 MRLs are posted on the ATSDR's web site (www.atsdr.cdc.gov/mrls/mrllist.asp; accessed September 24, 2010). For derivation of most of the MRLs, an uncertainty factor of 10 was used for interspecies extrapolation. However, for using dosimetric adjustments in cases involving inhalation exposures, many MRLs used a factor of 3 for extrapolation from animals to humans. The human equivalent concentration (HEC) was, for example, calculated in derivation of intermediate-duration inhalation MRL for chlorine gas [68]. The intermediate-duration inhalation MRL was calculated using EPA's methodology [69] for a category 1 gas:

$$\mathrm{LOAEL[HEC]} = \mathrm{LOAEL[ADJ]} \times \mathrm{RGDRTB}$$

where:
LOAEL[ADJ] = 0.5 ppm × 6/24 h × 5/7 days = 0.09 ppm (LOAEL: lowest-observable adverse effect level).
RGDRTB = (VE/SATB) animal/(VE/SATB) human (RGDRTB = ratio of the regional gas dose in rats to that of humans for the tracheobronchial region).
VE = minute volume (0.137 L/min for rats, 13.8 L/min for humans).
SATB = surface area of the tracheobronchial region (22.5 cm^2 for rats and 3,200 cm^2 for humans [69]).

Therefore, the HEC is:

$$\begin{aligned}\mathrm{LOAEL[HEC]} &= 0.09\,\mathrm{ppm} \times (0.137\,\mathrm{L/min}/22.5\,\mathrm{cm^2})/(13.8\,\mathrm{L/min}/3{,}200\,\mathrm{cm^2}) \\ &= 0.14\,\mathrm{ppm}\end{aligned}$$

Some oral MRLs used uncertainty factor of 3 instead of 10 for interspecies differences, as well. In these cases, a scientific judgment, rather than dosimetry,

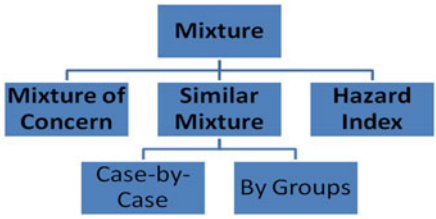


Fig. 1 Alternative approaches used for the toxicity assessment of mixtures (see text for explanations)

played a role in the adjustment. The database for chemicals such as ethion, hexachlorobenzene, and PCBs lended itself to such uncertainty factors application.

Chemical Mixtures

Typically, exposure to environmental chemicals is unintentional and is often to mixtures, simultaneously and/or sequentially. In public health practice, it is prudent to consider and ascertain that thresholds for harmful effects are not exceeded either individually or in combination. Consequently, for the risk assessment of mixtures, methods from single chemicals have been modified and advanced to include issues pertinent to mixtures. Three alternative approaches are available for the toxicity assessment of chemical mixtures, but each has its own shortcomings (Fig. 1) [70]. In general, the risk assessment community has accepted these approaches that are the basis of the various US government agency guidelines and international bodies such as EU and WHO. The most direct and accurate form of risk assessment, with least uncertainty, is possible when toxicological data are available for the "mixture of concern." If so, the data are reviewed and a criterion or regulatory standard is derived for occupational exposures and environmental mixtures (Tables 4 and 5). If data are unavailable for the mixture of concern but are available for a "similar mixture," they are used for derivation of a criterion after due considerations are given to the qualitative and quantitative aspects of the composition of the mixture. This approach is used on a case-by-case basis or by grouping of chemicals [71], since no criteria are set for the determination of a similar mixture. Risk assessment of a gasoline leak from an underground storage tank based on gasoline data would be an example of using similar mixture data. In both the above approaches, the mixture is treated as a single chemical because, to some extent, data are available on the whole mixture.

If data are not available on the whole mixture but are available for individual components of the mixtures, then a third approach, the "hazard index," is used. This, the most often used approach, is based on the concept of potency-weighted dose or response additivity. Through this procedure, an attempt is made to predict the toxicity of the whole mixture. The additivity assumption is valid only when there are no interactions among the components of the mixture through toxicokinetics or toxicodynamics phases and when the components elicit their toxic

Table 4 Threshold limit values (TLVs) for occupational exposures to complex mixtures [76]

Complex mixtures	TLV	Endpoint	Comments
Asphalt fumes	0.5 mg/m^3	Irritation	Inhalable fraction
Aliphatic hydrocarbon gases	1,000 ppm	CNS depression, cardiac sensitization	Alkanes (C_1–C_4)
Asbestos	0.1 f/cc	Asbestosis, cancer	Respirable fibers
Coal tar	0.2 mg/m^3	Cancer	As benzene soluble aerosol
Coal dust			
anthracite	0.4 mg/m^3	Lung fibrosis	Respirable fraction
bituminous	0.9 mg/m^3	Lung function	
Cotton dust	0.2 mg/m^3	Lung byssinosis	
Chromite ore	0.05 mg/m^3	Lung cancer	
Diesel fuel	100 mg/m^3	Skin irritation	Vapor
Flour dust	0.5 mg/m^3	Asthma	Inhalable fraction
Kerosene	200 mg/m^3	Skin, CNS, irritation	Total hydrocarbon vapor
Mica	3 mg/m^3	Pneumoconiosis	Respirable fraction
Mineral oil	5 mg/m^3	Lungs	Mist
Wood dust			
western red cedar	0.5 mg/m^3	Asthma	
all other species	1.0 mg/m^3	Pulmonary function	

Table 5 Minimal risk levels (MRLs) for mixtures of environmental contaminants [79]

Mixture	MRL value	Duration	Route
Fuel oil	0.02 mg/m^3	Acute	Inhalation
Jet fuel			
JP-4	9 mg/m^3	Intermediate	Inhalation
JP-7	0.3 mg/m^3	Chronic	Inhalation
JP-8	3 mg/m^3	Intermediate	Inhalation
Polybrominated biphenyls (PBBs)	0.01 mg/kg/day	Acute	Oral
Polychlorinated biphenyls (PCBs), aroclor 1254	0.03 µg/kg/day	Intermediate	Oral
	0.02 µg/kg/day	Chronic	Oral

response through similar mechanisms. Several environmental chemicals interact with each other by various mechanisms that are dependent on the dose, dosing regimen (i.e., single or repeated exposure), exposure pattern (i.e., simultaneous, staggered, pretreatment, coadministration, or sequential), and/or exposure route of one or all chemicals [72].

Examples of the No-Interaction Approach

This relates to treating the mixture as one entity and development of health guidance for the whole mixture. Similar to the approach used for the fuel oils,

ATSDR treated the exposure to a jet fuel as exposure to one entity. Exposure to jet fuel components, exhaust, or combustion products was not considered.

An MRL of 9 mg/m^3 was derived for intermediate-duration inhalation exposure to JP-4. The MRL was derived from a LOAEL of 500 mg/m^3 for increased hepatic toxicity in mice reported in a continuous exposure 90-day study [73]. The JP-4 concentration is based on the total hydrocarbon present in the vapors after the original mixture was heated to 50°C. The hepatic changes included vacuolization of cytoplasm of hepatocytes, mainly in the centrilobular region, and increased incidence of fatty degenerative changes. The LOAEL was adjusted to a HEC and divided by a UF of 300 (10 for use of a LOAEL, 3 for interspecies extrapolation, 10 for human variability).

An MRL of 0.3 mg/m^3 was derived for chronic-duration inhalation exposure to JP-7. The MRL was based on a LOAEL of 150 mg/m^3 that caused hepatic toxicity (inflammation) in female rats [74]. No such effects were observed in males. The dose was adjusted to a HEC and to continuous exposure and divided by a UF of 300 (10 for use of a LOAEL, 3 for interspecies extrapolation, 10 for human variability).

The second example uses the most toxic chemical from the mixture and provides a health guidance value based on the MRL for this chemical. Consequently, it uses toxicity equivalents (TEQs) to estimate the toxicity of the whole mixture. TEQ is defined as the concentration of an individual dioxin-like compound in a complex environmental mixture divided by the corresponding TCDD toxicity equivalency factor for that compound. The total TEQ is the sum of the TEQs for each of the congeners in a given mixture [46]. Assuming additivity of the toxic response, the generated total TEQ can be used to estimate the toxicity of an environmental mixture containing a known distribution of CDDs or CDFs relative to that of TCDD.

ATSDR's guidance values for TCDD were based on animal studies presented in the toxicological profile for CDDs [46]. For example, the chronic oral MRL of 1 pg/kg/day TCDD was based on a LOAEL for mild learning and behavioral impairment in the offspring of monkeys exposed to 0.00012 μg/kg/day TCDD in their feed [75]. An UF of 90 was used in the calculation of the MRL (3 for use of a minimal LOAEL, 3 for animal to human extrapolation, 10 for human variability). The MRL applies also to the total TEQs of the above mentioned related compounds.

Examples of the Interaction Approach

Presence of more than a single chemical can lead to interactions that can enhance, inhibit, or otherwise influence the toxicity of individual chemicals and thus modify the overall toxicity of the mixture (Table 4). Presence of multiple chemicals in limited compartments within the organs of the body increases the likelihood of interactions at pharmacokinetic and pharmacodynamic organizational levels. In fact, ample information supported by varying degrees of mechanistic understanding exists that substantiate interactions [77, 78]. Most toxicologists agree that this information should not be disregarded. However, there is less agreement and

limited recommendations on the use of this information in joint toxicity assessments. The potential significance of interactions in toxicity assessments can be regarded as one of many sources of uncertainty.

The uncertainty associated with interactions is somewhat difficult to characterize or quantify. One of the major reasons for this is that most of the information available on chemical interactions is not amenable to proper statistical and other types of analysis. A framework for systematically assessing the weight of evidence (WOE) for interactions has been adopted and used by several US federal agencies [78, 80, 81]. To some extent, this method provides means for the qualitative assessment of interactions (i.e., whether the mixture is likely to be more or less toxic than anticipated joint toxicity based on the assumption of additivity). Also, this method attempts to assess the magnitude of the interaction and quantitatively adjust the toxicity assessments using dose/response or dose/severity.

Briefly, the WOE evaluation is a qualitative judgment, based on empirical observations and mechanistic data. The method characterizes the plausibility of joint toxicity of pairs of toxicants, (i.e., how a chemical's toxicity can be influenced by the presence of a second toxicant). The WOE scheme yields an alphanumeric that takes into consideration several factors including the quality of the data, its mechanistic understanding (category I, II, III), its toxicological significance (category A, B, C), and factors such as route and duration of exposure that could play a critical role in the expression of the overall integrated toxicity of the mixture [80]. The application of this methodology is embedded in interaction profiles, a series of ATSDR documents, that evaluate and summarize the toxicity of the most frequently found chemicals mixtures in the environment. The unique characteristic that distinguished these summaries from mixtures reviews is it integrates the information on chemical interactions into the overall toxicity of the mixtures through the application of the WOE methodology [80]. These profiles also discuss how public health assessments can incorporate concerns about interactions, additivity, and potential human exposures to mixtures of chemicals.

One of the mixtures profiled was a mixture of chloroform, 1,1-dichloroethylene, trichloroethylene, and vinyl chloride [82]. These chemicals are among the top ten found in water at hazardous waste sites. They are at the 9th, 7th, 1st, and 8th most frequently found, respectively. Consequently, they are also encountered in combinations. For example, the binary combination of 1,1-dichloroethylene and trichloroethylene is the third most often found chemical in contaminated waters and was reported at 62 sites evaluated by ATSDR.

As part of the WOE methodology, potential binary pairs of chemicals are evaluated first and then conclusions for various endpoints are drawn for the overall mixture. The pairwise evaluation, an exposure-based screening assessment of potential health hazards for a binary mixture, is dependent upon the availability of the health effects and mechanistic data for the individual components of the mixture. Given below is the WOE evaluation of the influence of chloroform on trichloroethylene toxicity. It can be represented by the alphanumeric $<$ IA. Further detailed explanation follows:

Mechanistic understanding: The mechanism of interaction for hepatotoxicity is well understood. Many of the effects of chloroform are due to the formation of reactive intermediates, including phosgene, following metabolism by CYP enzymes,

particularly CYP2E1. Trichloroethylene is similarly metabolized by CYP2E1 to reactive intermediates and therefore could be hypothesized to compete for the enzyme at high exposure levels. In such a case, the toxicity of both compounds would be expected to be reduced, as insufficient amounts of the enzyme would be available to fully metabolize both chemicals. Analysis of the behavior of this interaction using a joint physiologically based toxicokinetic (PBPK) model developed for the two chemicals [83], using inhalation data in rats, indicated that the interaction was best modeled when competitive inhibition of the two compounds for the active site was assumed. Since a direct demonstration of the mechanism by which the interactions occurs exists, a rating of "I" was assigned.

Toxicological significance: Since the toxicological significance of the interaction was demonstrated in a hepatotoxicity study in rats using simultaneous acute intraperitoneal administration [84], a rating of "A" was assigned for hepatic effects.

Direction of interaction: Because both chloroform and trichloroethylene are metabolized to reactive metabolites by the same enzyme, the effects of each will be lessened due to a limitation on the rate of production of new metabolites. Hence, it was assigned the category $<$, i.e., less than additive. This has also been demonstrated in high-dose animal experimental studies. However, at low exposure levels, no interaction can be anticipated.

Thus, these types of analyses and findings provide the risk assessors information regarding the implications of species-specific toxicity information in risk assessment. If several pairs of chemicals of a mixture have a potential for greater than additive toxicity in a number of species, the final recommendation could include a concern for mixtures toxicity. While if a majority of pairs of a mixture have a potential for less than additive toxicity, there might not be a concern for mixtures in their final recommendations of an assessment.

Perspective

Apart from interspecies variation, a new series of uncertainties could be introduced in the risk assessment of chemicals. In today's world of a drive to reduce the use of animals in toxicity testing and an aggressive attempt to develop and adopt alternative testing methods, various *in vitro* methods are being evaluated for toxicity testing [85]. Some of these *in vitro* methods are based on human cell lines that could obtain direct data from human cells, rather than other species.

Risk assessors and public health officials should also be cognizant of aspects of variation within human populations. Apart from genetic polymorphism in biotransformation enzymes well documented in the literature [86], there is a new trend that could impact the human population gene pool. A genetic shift has been seen with increases in immigration from Asian countries to the USA. A "migration enabled gene flow" has contributed to a 3.6% increase in the overall pool. Such trends will continue to introduce interhuman variability.

References

1. Boxenbaum H (1982) Interspecies scaling, allometry, physiological time, and the ground plan of pharmacokientics. J Pharmacokinet Biopharm 10:201–227
2. Davidson WF, Parker JC, Beliles RP (1986) Biological basis for extrapolation across mammalian species. Regul Toxicol Pharmacol 6:211–237
3. Spiers DE, Candas V (1984) Relationship of skin surface area to body surface in the immature rat: a reexamination. J Appl Physiol 56:240–243
4. O'Flaherty EJ (1989) Interspecies conversion of kinetically equivalent doses. Risk Anal 9: 587–598
5. Bardodej Z, Bardodejova E (1970) Biotransformation of ethylbenzene, styrene, and α-methylstyrene in man. Am Ind Hyg Assoc J 31:206–209
6. Climie IJG, Hutson DH, Stoydin G (1983) The metabolism of ethylbenzene hydroperoxide in the rat. Xenobiotica 13:611–618
7. Engstrom K, Elovaara E, Aitio A (1985) Metabolism of ethylbenzene in the rat during long-term intermittent inhalation exposure. Xenobiotica 15:281–286
8. Engstrom K, Riihimaki V, Laine A (1984) Urinary disposition of ethylbenzene and *m*-xylene in man following separate and combined exposure. Int Arch Occup Environ Health 54:355–363
9. El Masri AM, Smith JN, Williams RT (1958) The metabolism of alkylbenzenes: phenylacetylene and phenylethylene (styrene). Biochem J 68:199–204
10. El Masri AM, Smith JN, Williams RT (1956) The metabolism of alkylbenzenes: *n*-Propylbenzene and *n*-butylbenzene with further observations on ethylbenzene. Biochem J 64:50–56
11. Smith JN, Smithies RH, Williams RT (1954) The metabolism of alkylbenzenes. Stereochemical aspects of the biological hydroxylation of ethylbenzene to methylphenylcarbinol. Biochem J 56:320–324
12. Smith JN, Smithies RH, Williams RT (1954) The metabolism of alkylbenzenes. (a) Glucuronic acid excretion following the administration of alkylbenzenes. (b) Elimination of toluene in the expired air of rabbits. Biochem J 56:317–320
13. Cruzan G, Carlson GP, Johnson KA (2002) Styrene respiratory tract toxicity and mouse lung tumors are mediated by CYP2F-generated metabolites. Regul Toxicol Pharmacol 35:308–319
14. IARC (2002) Some traditional herbal medicines, some mycotoxins, naphthalene and styrene, vol 82, IARC monographs on the evaluation of carcinogenic risk to humans. International Agency for Research on Cancer (IARC), Lyon, France, pp 437–550
15. Sumner SJ, Fennell TR (1994) Review of the metabolic fate of styrene. Crit Rev Toxicol 24 (Suppl 1):S11–S33
16. Cruzan G, Carlson GP, Turner M, Mellert W (2005) Ring-oxidized metabolites of styrene contribute to styrene-induced Clara-cell toxicity in mice. J Toxicol Environ Health 68A: 229–237
17. De Palma G, Manini P, Mozzoni P, Andreoli R, Bergamaschi E, Cavazzini S, Franchini I, Mutti A (2001) Polymorphism of xenobiotic-metabolizing enzymes and excretion of styrene-specific mercapturic acids. Chem Res Toxicol 14:1393–1400
18. Manini P, Buzio L, Andreoli R, Goldoni M, Bergamaschi E, Jakubowski M, Vodicka P, Hirvonen A, Mutti A (2003) Assessment of biotransformation of the arene moiety of styrene in volunteers and occupationally exposed workers. Toxicol Appl Pharmacol 189:160–169
19. Manini P, Andreoli R, Poli D, De Palma G, Mutti A, Niessen WM (2002) Liquid chromatography/electrospray tandem mass spectrometry characterization of styrene metabolism in man and in rat. Rapid Commun Mass Spectrom 16:2239–2248
20. Cruzan G, Faber WD, Johnson KA, Roberts LS, Hellwig J, Maurissen J, Beck MJ, Radovsky A, Stump DG (2005) Developmental neurotoxicity study of styrene by inhalation in Crl-CD rats. Birth Defects Res B Dev Reprod Toxicol 74:221–232
21. Nakajima T, Elovaara E, Gonzalez FJ, Gelboin HV, Vainio H, Aoyama T (1993) Characterization of the human cytochrome P450 isozymes responsible for styrene metabolism. IARC Sci Publ 101

22. Kim H, Wang RS, Elovaara E, Raunio H, Pelkonen O, Aoyama T, Vainio H, Nakajima T (1997) Cytochrome P450 isozymes responsible for the metabolism of toluene and styrene in human liver microsomes. Xenobiotica 27:657–665
23. Carlson GP, Mantick NA, Powley MW (2000) Metabolism of styrene by human liver and lung. J Toxicol Environ Health A 59:591–595
24. Nakajima T, Wang RS, Elovaara E, Gonzalez FJ, Gelboin HV, Vainio H, Aoyama T (1994) CYP2C11 and CYP2B1 are major cytochrome P450 forms involved in styrene oxidation in liver and lung microsomes from untreated rats, respectively. Biochem Pharmacol 48:637–642
25. Hynes DE, DeNicola DB, Carlson GP (1999) Metabolism of styrene by mouse and rat isolated lung cells. Toxicol Sci 51:195–201
26. Mendrala AL, Langvardt PW, Nitschke KD, Quast JF, Nolan RJ (1993) *In vitro* kinetics of styrene and styrene oxide metabolism in rat, mouse, and human. Arch Toxicol 67:18–27
27. Allis JW, Brown BL, Zhao G, Pegram RA (2001) The effects of inhalation exposure to bromodichloromethane on specific rat CYP isoenzymes. Toxicology 161:67–77
28. Pankow D, Damme B, Wunscher U, Bergmann K (1997) Chlorodibromomethane metabolism to bromide and carbon monoxide in rats. Arch Toxicol 71:203–210
29. Juchau MR, Boutelet-Bouchan H, Huang Y (1998) Cytochrome-P-450-dependent biotransformation of xenobiotics in human and rodent embryonic tissues. Drug Metab Rev 30: 541–568
30. Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC, Nebert DW (1996) P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. Pharmacogenetics 6:1–42
31. Czerwinski M, McLemore T, Gelboin H, Gonzalez FJ (1994) Quantification of CYP2B7, CY4B1, and CYPOR messenger RNAs in normal lung and lung tumors. Cancer Res 54: 1085–1091
32. Mehendale HM (1994) Amplified interactive toxicity of chemicals at nontoxic levels: mechanistic considerations and implications to public health. Environ Health Perspect 102(Suppl 9):139–149
33. Kulkarni SG, Duong H, Gomila R, Mehendale HM (1996) Strain differences in tissue repair response to 1,2-dichlorobenzene. Arch Toxicol 70:714–723
34. Kulkarni SG, Warbritton A, Bucci TJ, Mehendale HM (1997) Antimitotic intervention with colchicine alters the outcome of *o*-DCB-induced hepatotoxicity in Fischer 344 rats. Toxicology 120:79–88
35. Dalu A, Mehendale HM (1996) Efficient tissue repair underlies the resiliency of postnatally developing rats to chlordecone + CCl_4 hepatotoxicity. Toxicology 111:29–42
36. Shankar K, Vaidya VS, Apte UM, Manautou JE, Ronis MJ, Bucci TJ, Mehendale HM (2003) Type 1 diabetic mice are protected from acetaminophen hepatotoxicity. Toxicol Sci 73:220–234
37. Shankar K, Vaidya VS, Wang T, Bucci TJ, Mehendale HM (2003) Streptozotocin-induced diabetic mice are resistant to lethal effects of thioacetamide hepatotoxicity. Toxicol Appl Pharmacol 188:122–134
38. Wang T, Fontenot RD, Soni MG, Bucci TJ, Mehendale HM (2000) Enhanced hepatotoxicity and toxic outcome of thioacetamide in streptozotocin-induced diabetic rats. Toxicol Appl Pharmacol 166:92–100
39. Wang T, Shankar K, Bucci TJ, Warbritton A, Mehendale HM (2001) Diallyl sulfide inhibition of CYP2E1 does not rescue diabetic rats from thioacetamide-induced mortality. Toxicol Appl Pharmacol 173:27–37
40. Dalu A, Cronin GM, Lyn-Cook BD, Mehendale HM (1995) Age-related differences in TGF-α and proto-oncogenes expression in rat liver after a low dose of carbon tetrachloride. J Biochem Toxicol 10:259–264
41. Dalu A, Warbritton A, Bucci TJ, Mehendale HM (1995) Age-related susceptibility to chlordecone-potentiated carbon tetrachloride hepatotoxicity and lethality is due to hepatic quiescence. Pediatr Res 38:140–148
42. Murali B, Korrapati MC, Warbritton A, Latendresse JR, Mehendale HM (2002) Tolerance of aged Fischer 344 rats against chlordecone-amplified carbon tetrachloride toxicity. Mech Ageing Dev 125:421–435

43. Cai ZW, Mehendale HM (1990) Lethal effects of CCl_4 and its metabolism by Mongolian gerbils pretreated with chlordecone, phenobarbital, or mirex. Toxicol Appl Pharmacol 104:511–520
44. Stine ER, Gunawardhana L, Sipes IG (1991) The acute hepatotoxicity of the isomers of dichlorobenzene in Fischer-344 and Sprague–Dawley rats: isomer-specific and strain-specific differential toxicity. Toxicol Appl Pharmacol 109:472–481
45. Cortopassi GA, Wang E (1996) There is substantial agreement among interspecies estimates of DNA repair activity. Mech Ageing Dev 91:211–218
46. ATSDR (1998) Toxicological profile for chlorinated dibenzo-*p*-dioxins. Agency for toxic substances and disease registry, US Department of Health and Human Services, Atlanta, GA
47. Poland A, Glover E, Kende AS (1976) Stereospecific, high affinity binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin by hepatic cytosol: evidence that the binding species is a receptor for induction of aryl hydrocarbon hydroxylase. J Biol Chem 251:4936–4946
48. Okey AB, Riddick DS, Harper PA (1994) The Ah receptor: mediator of the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds. Toxicol Lett 70:1–22
49. Okey AB, Vella LM, Harper PA (1989) Detection and characterization of a low affinity form of cytosolic Ah receptor in livers of mice nonresponsive to induction of cytochrome P1-450 by 3-methylcholanthrene. Mol Pharmacol 35:823–830
50. Whitlock JP Jr (1993) Mechanistic aspects of dioxin action. Chem Res Toxicol 6:754–763
51. Hankinson O (1995) The aryl hydrocarbon receptor complex. Annu Rev Pharmacol Toxicol 35:307–340
52. Mason G, Safe S (1986) Synthesis, biologic and toxic effects of the major 2,3,7,8-tetrachlorodibenzo-*p*-dioxin metabolites in the rat. Toxicology 41:153–159
53. Poland A, Glover E (1979) An estimate of the maximum *in vivo* covalent binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to rat liver protein, ribosomal RNA and DNA. Cancer Res 39:3341–3344
54. Gonzales FJ, Fernandez-Salguero P (1998) The aryl hydrocarbon receptor: studies using the AHR-null mice. Drug Metab Dispos 26:1194–1198
55. Vorderstrasse BA, Stephan LB, Silvestrone AE, Kerkvliet NI (2001) Aryl hydrocarbon receptor-deficient mice generate normal immune responses to model antigens and are resistant to TCDD-induced immune suppression. Toxicol Appl Pharmacol 171:157–164
56. Pohjanvirta R, Viluksela M, Tuomisto JT, Unkila M, Karasinska J, Franc MA, Holowenko M, Giannone JV, Harper PA, Tuomisto J, Okey AB (1999) Physicochemical differences in the Ah receptors of the most TCDD-susceptible and the most TCDD-resistant rat strains. Toxicol Appl Pharmacol 155:82–95
57. Bunger MK, Moran SM, Glover E, Thomae TL, Lahvis GP, Lin BC, Bradfield CA (2003) Resistance to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxicity and abnormal liver development in mice carrying a mutation in the nuclear localization sequence of the aryl hydrocarbon receptor. J Biol Chem 278:17767–17774
58. Walisser JA, Bumger MK, Glover E, Harstad EB, Bradfield CA (2004) Patent ductus venosus and dioxin resistance in mice harboring a hypomorphic Arnt allele. J Biol Chem 279: 16326–16331
59. Hamadeh HK, Bushel P, Tucker CJ, Martin K, Paules R, Afshari CA (2002) Detection of diluted gene expression alterations using cDNA microarrays. Biotechniques 32: 322, 324, 326–329
60. Waring JF, Ciurlionis R, Jolly RA, Heindel M, Ulrich RG (2001) Microarray analysis of hepatotoxins *in vitro* reveals a correlation between gene expression profiles and mechanisms of toxicity. Toxicol Lett 120:359–368
61. Waring JF, Cavet G, Jolly RA, McDowell J, Dai H, Ciurlionis R, Zhang C, Stoughton R, Lum P, Ferguson A, Roberts CJ, Ulrich RG (2003) Development of a DNA microarray for toxicology based on hepatotoxin-regulated sequences. EHP Toxicogenomics 111:53–60
62. Bovenhof DR, Burgoon LD, Tashiro C, Sharratt B, Chittim B, Harkema JR, Mendrick DL, Zacharewski TR (2006) Comparative toxicogenomic analysis of the hepatotoxic effects of TCDD in Sprague–Dawley rats and C57BL/6 mice. Toxicol Sci 94:398–416

63. Dourson ML, Stara JF (1983) Regulatory history and experimental support of uncertainty (safety) factors. Regul Toxicol Pharmacol 3:224–238
64. Lu FC (1983) Toxicological evaluations of carcinogens and noncarcinogens: pros and cons of different approaches. Regul Toxicol Pharmocol 3:121–132
65. Lipscomb JC, Kedderis GL (2002) Incorporating human interindividual biotransformation variance in health risk assessment. Sci Total Environ 288:13–21
66. Renwick AG (1993) Data-derived safety factors for the evaluation of food additives and environmental contaminants. Food Addit Contam 10:275–305
67. WHO (2005) Chemical-specific adjustment factors for interspecies differences and human variability: guidance document for use of data in dose/concentration-response assessment. World Health Organization, Geneva, Switzerland
68. ATSDR (2007) Toxicological profile for chlorine. Agency for Toxic Substances and Disease Registry, US Department of Health and Human Services, Atlanta, GA
69. US-EPA (1994) Methods for derivation of inhalation reference concentrations and application of inhalation dosimetry. US Environmental Protection Agency, Office of Research and Development, Washington DC
70. ATSDR (2001) Guidance manual for the assessment of joint toxic action of chemical mixtures. Agency for Toxic Substances and Disease Registry, US Department of Health and Human Services, Atlanta, Georgia, USA, Online available at http://www.atsdr.cdc.gov/interactionprofiles
71. Durkin P, Hertzberg R, Stiteler W, Mumtaz MM (1995) The identification and testing of interaction patterns. Toxicol Lett 79:251–264
72. Krishnan K, Brodeur J (1991) Toxicological consequences of combined exposure to environmental pollutants. Arch Complex Environ Studies 3:1–106
73. Air Force (1984) Ninety-day continuous inhalation exposure to petroleum JP-4 jet fuel. In: Toxic hazards research unit annual technical report no. AMRL-TR-84-001. Wright-Patterson Air Force Base, OH, Aerospace Medical Research Laboratory, Aerospace Medical Division, Air Force Systems Command, document no. AD-A147857/7, 46–62
74. Air Force (1991) Tumorigenic evaluation of jet fuels JP-TS and JP-7. Report no AAMRL-TR-91-0020. Wright-Patterson Air Force Base, OH, Aerospace Medical Research Laboratory, Aerospace Medical Division, Air Force Systems Command
75. Schantz SL, Ferguson SA, Bowman RE (1992) Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on behavior of monkey in peer groups. Neurotoxicol Teratol 14:433–446
76. ACGIH (2010) Annual reports of the committees on TLVs® and BEIs®. American Conference of Governmental and Industrial Hygienists, Wright-Patterson AFB, OH. Online available at http://www.acgih.org/TLV/
77. Mumtaz MM, Hertzberg RC (1993) The status of interactions data in risk assessment of chemical mixtures. In: Saxena J (ed) Hazard assessment of chemicals, vol 8. Hemisphere Publishing Corporation, Washington DC, pp 47–79
78. De Rosa CT, El-Masri HE, Pohl H, Cibulas W, Mumtaz MM (2004) Implications of chemical mixtures for public health practice. J Toxicol Environ Health 7:339–350
79. ATSDR (2010) Minimal risk levels (MRLs) for hazardous substances. Agency for Toxic Substances and Disease Registry, US Department of Health and Human Services, Atlanta, Georgia. Online available at http://www.atsdr.cdc.gov/mrls/mrllist.asp
80. Mumtaz MM, Durkin PR (1992) A weight-of-evidence approach for assessing interactions in chemical mixtures. Toxicol Ind Health 8:377–406
81. Pohl HR, Mumtaz MM, Scinicariello F, Hansen H (2009) Binary weight-of-evidence evaluations of chemical interactions—15 years of experience. Regul Toxicol Pharmacol 54: 264–271
82. ATSDR (2010) Interaction profile for chloroform, 1,1-dichloroethylene, trichloroethylene, and vinyl chloride. Agency for Toxic Substances and Disease Registry, US Department of Health and Human Services, Atlanta, Georgia. Online available at http://www.atsdr.cdc.gov/interactionprofiles/index.asp

83. Isaacs KK, Evans MV, Harris TR (2004) Visualization-based analysis for a mixed-inhibition binary PBPK model: determination of inhibition mechanism. J Pharmacokinet Pharmacodyn 31:215–242
84. Anand SS, Mumtaz MM, Mehendale HM (2005) Dose-dependent liver tissue repair after chloroform plus trichloroethylene binary mixture. Basic Clin Pharmacol Toxicol 96:436–444
85. ICCVAM (2010) U.S. and international acceptance of alternative methods, 1998–2011. National Institutes of Environmental Health Sciences (NIEHS), National Toxicology Program (USA), Research Triangle Park, NC. Online available at http://iccvam.niehs.nih.gov/about/about_ICCVAM.htm
86. Enomoto N, Takase S, Yasuhara M, Takada A (1991) Acetaldehyde metabolism in different aldehyde dehydrogenase-2 genotypes. Alcohol Clin Exp Res 15:141–144

Recent Trends in Statistical QSAR Modeling of Environmental Chemical Toxicity

Alexander Tropsha

Abstract Quantitative cheminformatics approaches such as QSAR modeling find growing applications in chemical risk assessment. Traditional methods rely on the use of calculated chemical descriptors of molecules and relatively small training sets. However, in recent years, there is a trend toward the increased use of *in vitro* biological testing approaches to reduce both the length of experimental studies and the animal use for chemical risk assessment. Furthermore, there is also much greater emphasis on model validation using external datasets to enable the reliable use of computational models as part of regulatory decision making. In this chapter, recent trends emphasizing the need for both careful curation of experimental data prior to model development and rigorous model validation are investigated. Furthermore, recent approaches to chemical toxicity prediction that employ both chemical descriptors and *in vitro* screening data for developing novel hybrid chemical/biological models are being reviewed. Examples of respective application studies that employ novel workflows for model developments are described and recent important efforts by several academic, nonprofit, and industrial groups to start placing both data and, especially, models in the public domain are discussed.

Keywords Cheminformatics · Quantitative structure–activity relationships · Hybrid chemical–biological descriptors · Quantitative chemical health risk assessment · Regulatory decision support

Alexander Tropsha (✉)
Laboratory of Molecular Modeling, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA
e-mail: alex_tropsha@unc.edu

A. Luch (ed.), *Molecular, Clinical and Environmental Toxicology*,
Experientia Supplementum 101, DOI 10.1007/978-3-7643-8340-4_13,

Introduction

There is a critical need to develop alternative means to *in vivo* toxicity testing of chemical compounds. In a guiding effort to advance toxicity testing in the twenty-first century, the National Research Council recently outlined a new vision and strategies for the increased use of *in vitro* and computational technologies for chemical risk assessment [1, 2]. To this end, there have been recent rapid developments of multiple short-term assay panels for parallel screening of large collections of chemicals that may present health risks. These assays are expected to substitute, at least partially, the *in vivo* animal studies [3], and the ToxCast™ project at the Environmental Protection Agency (EPA) represents a major public effort in this direction [4]. At the same time, novel *in silico* approaches are finding an increased utility in regulatory chemical risk assessment [5, 6].

In silico predictive toxicology models fall into two principal categories: rule-based (expert) systems and statistical QSAR modeling. Rule-based systems are built upon the earlier work of Miller [7] and others [8–11], who first systematized relationships between chemical substructures and observed toxic outcomes (rules). Such rules are favored by toxicologists because of their perceived transparency in the form of structure alerts. However, the designation of a substructure or fragment as a toxicophore is only a binary indication. The steric and electronic environment of a structural alert can diminish its toxic potency [12], render a fragment nontoxic, or create a new toxic fragment.

QSAR approaches predict compounds' properties solely from molecular structures as defined by their chemical descriptors. The QSAR-based computational toxicity tools have been used to assist in predictive toxicological profiling of pharmaceutical substances to identify potential drug safety liabilities [13–16], evaluate the chemical safety risks by pharmaceutical companies and environmental agencies [14, 17], and support regulatory decision making concerning chemical safety and toxicity risk assessment [18], as well as effectively enhance an already rigorous US regulatory review of pharmaceutical substances' safety [19].

This wide and perhaps growing use of predictive QSAR toxicity models notwithstanding, many studies have also suggested that current tools do not work well to evaluate *in vivo* toxicity potentials, especially for compounds that were not used for model development [6, 20]. Thus, addressing the challenges outlined in aforementioned National Academy of Sciences report requires the development of robust and mechanistically relevant short-term biological assays as well as reliable, externally predictive computational models. Traditional *in silico* approaches for predicting chemical effects *in vivo* typically either rely on the use of calculated physical properties or structural features of chemicals (i.e., chemical descriptors) employed in quantitative structure–activity/property relationship (QSAR/QSPR)[1] modeling or attempt to relate the results of short-term assays to the *in vivo* outcome of chemical

[1]In this paper, we shell use the terms QSAR and QSPR interchangeably

exposure (*in vitro–in vivo* correlations). Although both types of approaches have clear merits, the existing tools and models should be substantially improved to enable both their reliable application for prioritizing compounds for experimental toxicity testing and their use in chemical safety regulations. In fact, both types of efforts are complementary and should be explored in parallel. Furthermore, as discussed in this chapter, short-term assay data can be easily integrated with cheminformatics approaches to improve the accuracy of *in vivo* toxicity prediction.

It must be recognized that the ultimate *success* of computational toxicity models developed with statistical QSAR approaches depends on *three major factors*: (1) the accuracy of primary data used for model development, (2) the rigor of modeling approaches and computational tools employed to analyze the data, and (3) the availability and the ease of use of computational tools and models. In this chapter, recent trends in statistical QSAR modeling of chemical toxicity in the context of these major contributing factors are discussed. Specifically, following the general introduction of major QSAR modeling approaches for achieving rigorous, validated, and externally predictive models the following will be discussed: the need and protocols for thorough curation of the experimental data on chemical structure as well as *in vitro* and/or *in vivo* activity of chemicals in historic datasets; application of novel data-analytical procedures that exploit both computed chemical descriptors and experimental data resulting from short-term biological assays for developing enhanced models of *in vivo* chemical toxicity; and the delivery of integrated chemical toxicity databases, tools, and models, in the user-friendly format, e.g., via specialized web portals. The current trends in statistical QSAR modeling of chemical toxicity explored in this chapter indicate the growing use of *in silico* toxicity prediction approaches for prioritizing both existing and novel environmental chemicals for in-depth toxicity screening and for regulatory chemical risk assessment.

Brief Notes on the QSAR Modeling Methodology

In an important paper that was somewhat provocatively titled "*In silico* ADME/Tox: why models fail," Stouch *et al*. [21] evaluated several commercially available ADME/Tox predictors and attempted to understand the causes of their frequent failures. Often, in their observation, the interpretation of the success of the models lies in their use and the expectations of the user. Disappointing results could be linked to the key aspects of the modeling procedures, many of which related to the original data and their interpretation. Lombardo *et al*. [22] suggested that not much progress has been made in developing robust and predictive models, and that the lack of accurate data, together with the use of questionable modeling endpoints, has hindered the real progress. These observations suggest that there is a continuing need to develop novel QSAR modeling and data treatment procedures to improve the outcome of the QSAR modeling of chemical toxicity.

Modern QSAR modeling is a very complex and complicated field requiring deep understanding and thorough practicing to develop robust models. Multiple types of chemical descriptors and numerous statistical approaches to model development can

be found in the specialized literature, and so they need not be discussed in this chapter. Instead, several unifying concepts that underlie practically any QSAR methodology are being presented.

Any QSAR method can be generally defined as an application of mathematical and statistical methods to the problem of finding empirical relationships (QSAR models) of the form $P_i = k\text{^}(D_1, D_2, \ldots D_n)$, where P_i are biological activities (or other properties of interest) of molecules, $D_1, D_2, \ldots, D_n$ are calculated (or, sometimes, experimentally measured) structural properties (molecular descriptors) of compounds, and k^ is some empirically established mathematical transformation that should be applied to descriptors to calculate the property values for all molecules. The relationship between values of descriptors D and target properties P can be linear or nonlinear. The example of the former relationship is given by multiple linear regression (MLR) common to the Hansch QSAR approach [23], where target property can be predicted directly from the descriptor values. On the contrary, nearest neighbor QSAR methods (e.g., [24]) serve as examples of nonlinear techniques where descriptor values are used in characterizing chemical similarities between molecules, which are then used to infer compound activity. The goal of QSAR modeling is to establish a trend in the descriptor values, which parallels the trend in biological activity. In essence, all QSAR approaches imply, directly or indirectly, a simple similarity principle, which for a long time has provided a foundation for the experimental medicinal chemistry, i.e., compounds with similar structures are expected to have similar biological activities.

Modern QSAR approaches are characterized by the use of multiple descriptors of chemical structure combined with the application of both linear and nonlinear optimization approaches, and a strong emphasis on rigorous model validation to afford robust and predictive models. As mentioned above, the most important recent developments in the field concur with a substantial increase in the size of experimental datasets available for the analysis and an increased application of QSAR models as virtual screening tools to discover biologically active molecules in chemical databases and/or virtual chemical libraries [25]. The latter focus differs substantially from the traditional emphasis on developing so-called explanatory QSAR models characterized by high statistical significance but only as applied to training sets of molecules with known chemical structure and biological activity.

The differences in various QSAR methodologies can be understood in terms of the types of *target property* values, *descriptors*, and *optimization* algorithms used to relate descriptors to the target properties and generate statistically significant models. *Target* properties (regarded as dependent variables in statistical data modeling sense) can be generally of three types: *continuous* (i.e., real values covering certain range, e.g., IC_{50} values, or binding constants), *categorical related or rank-based* (e.g., classes of rank-ordered target properties covering certain range of values, e.g., classes of metabolic stability such as unstable, moderately stable, stable), and *categorical unrelated* (i.e., classes of target properties that do not relate to each other in any continuum, e.g., compounds that belong to different pharmacological classes). As simple as it appears, understanding this classification is actually very important since the choice of descriptor types and modeling techniques as well

as model accuracy metrics is often dictated by the type of the target properties. Thus, in general, the latter two types require *classification* modeling approaches whereas the former type of the target properties allows the use of (multi)linear regression type modeling. The corresponding methods of data analysis are referred to as either classification or continuous property QSAR.

Many QSAR approaches have been developed during the past few decades (e.g., see recent reviews [26, 27]). The major differences between various approaches are due to structural parameters (descriptors) used to characterize molecules and the mathematical approaches used to establish a correlation between descriptor values and biological activity. Most of the modeling techniques assume a linear relationship between molecular descriptors and a target property, which may be an adequate methodology for many datasets. However, the advances in combinatorial chemistry and high throughput screening technologies have resulted in the explosive growth of the amount of structural and biological data, making the problem of developing robust QSAR models more challenging. This progress has provided an impetus for the development of fast, nonlinear QSAR methods that can capture structure–activity relationships for large and complex data. New nonlinear methods of multivariate analysis such as different types of artificial neural networks [28–31], generalized linear models [29, 32–34], classification and regression trees [32, 35–38], random forests [39–41], MARS (multivariate adaptive regression splines) [41, 42], support vector machines [43–46], and some other methods have become routine tools in QSAR studies. Interesting examples of applications have been reported for all types of the above methods. In some cases, the comparisons between different techniques as applied to the same dataset have been made, but in general, there appears to be no universal QSAR approach that produces the best QSAR models for any datasets. To this end, we have always favored a combinatorial QSAR approach to model development where various combinations of data modeling methods and different types of chemical descriptors are explored to achieve models of the highest rigor and external predictive power [47–50].

Recent research has also emphasized model validation as the key component of QSAR modeling [51]. Specifically, we [51, 52] and others [53–56] have demonstrated that various, commonly accepted, statistical characteristics of QSAR models derived for a training set are insufficient to establish and estimate the predictive power of quantitative structure–toxicity relationship (QSTR) models.

Critical Importance of QSAR Model Validation

It should sound almost axiomatic that validation should be natural part of any model development process. Indeed, what is the (ultimate) purpose of any modeling approach such as QSAR, if not developing models with a significant external predictive power? Unfortunately, as we and others have indicated in many publications (e.g., [51, 55, 57]), the field of QSAR modeling has been plagued with insufficient attention paid to the subject of external validation. Indeed, most practitioners have merely

presumed that internally cross-validated models built from available training set data should be externally predictive. As mentioned in the Introduction, the large number of QSAR publications exploring small- to medium-size datasets to produce models with little statistical significance led to the editorial published by the *Journal of Chemical Information and Modeling* recently [58] that explicitly discouraged researchers from submitting the "introspective" QSAR/QSPR publications and requested that "evidence that any reported QSAR/QSPR model has been properly validated using data not in the training set must be provided." We and others have demonstrated (as detailed below) that the training set statistics using most common internal validation techniques such as leave-one-out or even leave-many-out cross-validation approaches is insufficient, and the statistical figures of merit of such models serve as misleading indicators of the external predictive power of QSAR models [51].

In our highly cited publication "Beware of q^2!" [57], we have demonstrated the insufficiency of the training set statistics for developing externally predictive QSAR models and formulated the main principles of model validation. At the time of that publication in 2002, the majority of papers on QSAR analysis ignored any model validation except for the cross-validation, performed during model development. Despite earlier observations of several authors [53, 56, 59] warning that high cross-validated correlation coefficient R^2 (q^2) is the necessary, but not the sufficient condition for the model to have high predictive power, many authors continued to consider q^2 as the only parameter characterizing the predictive power of QSAR models. In [57], we have shown that the predictive power of QSAR models can be claimed only if the model was successfully applied for prediction of the external test set compounds, which were not used in the model development. We have demonstrated that the majority of the models with high q^2 values have poor predictive power when applied for prediction of compounds in the external test set. In another publication [51], the importance of rigorous validation was again emphasized as a crucial, integral component of model development. Several examples of published QSPR models with high fitted accuracy for the training sets, which failed rigorous validation tests, have been considered. We presented a set of simple guidelines for developing validated and predictive QSPR models and discussed several validation strategies such as the randomization of the response variable (*Y*-randomization) external validation using rational division of a dataset into training and test sets. We highlighted the need to establish the domain of model applicability in the chemical space to flag molecules for which predictions may be unreliable and discussed some algorithms that can be used for this purpose. We advocated the broad use of these guidelines in the development of predictive QSPR models [51, 52, 60].

I shall also comment on the issue of so-called mechanistic QSAR. Some authors (e.g., [61]) prefer descriptors which are mechanistically interpretable. On the other hand, Estrada and Patlewicz [62] argued that in many cases a biological response is a result of a multitude of different processes, some of which can be even not known, and the a posteriori mechanistic interpretation of a complex process is difficult if not impossible. The authors suggested an alternative approach where a biological system is considered as a black box, when considering several possible mechanisms

would be more productive. At the same time, some variables included in the model can describe several different mechanisms simultaneously, for instance, log *P*, so in many cases, it makes no sense to suggest that the use of this descriptor in QSAR models affords any mechanistic interpretation (see also [63]). We would add that descriptors which give better models in terms of their predictive power are actually preferable. We consider building predictive models as the main goal of QSAR analysis. Of course, the interpretation of the model is also important, and if possible, it should be done. However, in many cases, it is impossible, even when models with high predictive power have been obtained (e.g., best models were found to be those using the molecular connectivity indices, but these models were disregarded by the authors for the lack of mechanistic interpretability [61]). We believe that mechanistic interpretation of the *externally validated* QSAR model is an important *a posteriori* exercise that should be done after the model has been internally and externally validated, and descriptors that afford models with the highest predictive power should be always used preferentially.

The need for a rigorous validation of QSAR models has been increasingly recognized by QSAR practitioners as well as regulatory agencies. At the 37th Joint Meeting of Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology, held in Paris on November 17–19, 2004, the OECD (Organization for Economic Co-operation and Development) member countries adopted the following five principles that valid (Q)SAR models should follow to allow their use in regulatory assessment of chemical safety [64, 65]: (1) a defined endpoint; (2) an unambiguous algorithm; (3) a defined domain of applicability; (4) appropriate measures of goodness-of-fit, robustness, and predictivity; and (5) a mechanistic interpretation, if possible. Since then, most of the European authors publishing in QSAR area include a statement that their models fully comply with OECD principles (e.g., see [66–69]).

Validation of QSAR models remains one of the most critical problems of QSAR. Recently, we have extended our requirements for the validation of multiple QSAR models selected by acceptable statistics criteria of prediction of the test set [70]. Additional studies in this critical component of QSAR modeling should establish reliable and commonly accepted "good practices" for model development. In a recent review, we have summarized our views of best QSAR modeling practices [71] that have been incorporated in the modeling workflow that is briefly reviewed below.

Predictive QSAR Modeling Workflow

In our opinion, the QSAR modeling process should not be restricted to building models of existing training sets but should include extensive and rigorous model validation, both internal and external. Following this paradigm, in recent years, we have developed [71, 72] and implemented in a publicly available web portal [73] a QSAR modeling workflow that is summarized in Fig. 1. The workflow focuses on delivering validated models and computational hits that should be ultimately confirmed by the experimental validation. In brief, we start by carefully curating chemical

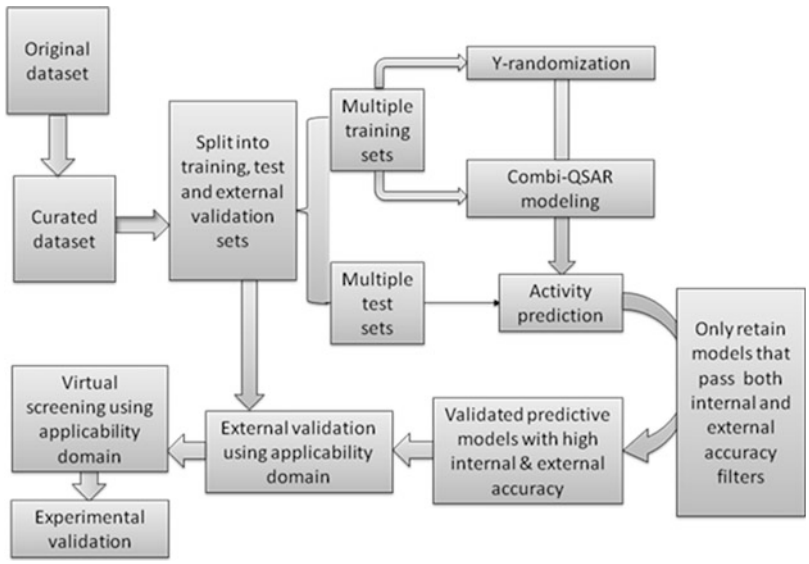


Fig. 1 Flowchart of predictive toxicology framework based on validated QSAR/QSTR models

structures and, if possible, associated biological activities to prepare the dataset for subsequent calculations (see additional discussion of this critical component below). Then, a fraction of compounds (typically, 10–20%) is selected randomly as an external evaluation set. A more rigorous *n*-fold external validation protocol can be employed when the dataset is randomly divided into *n* nearly equal parts, and then, $n - 1$ parts are systematically used for model development, and the remaining fraction of compounds is used for model evaluation. The sphere exclusion protocol implemented in our laboratory [60, 74] is then used to rationally divide the remaining subset of compounds (the modeling set) into multiple training and test sets that are used for model development and validation, respectively. We employ multiple QSAR techniques based on the combinatorial exploration of all possible pairs of descriptor sets and various supervised data analysis techniques (combi-QSAR) and select models characterized by high accuracy in predicting both training and test sets data. The model acceptability thresholds are typically characterized by the lowest acceptable value of the leave-one-out, cross-validated R^2 (q^2) for the training set and by the conventional R^2 for the test set; our default values are 0.6 for both q^2 and R^2. All validated models are finally tested in an ensemble using the external evaluation set.

The critical step of the external validation is the use of applicability domains (AD), which are defined uniquely for each model used in consensus (ensemble) prediction of the external set. If external validation demonstrates the significant predictive power of the models, we employ them for virtual screening of chemical datasets of interest such as environmental chemicals of concern identified by REACH. The entire approach is described in detail elsewhere [71, 72]. Models resulting from this workflow could be used to prioritize the selection of chemicals for the experimental validation.

Chemical Toxicity Data and the Need for Data Curation Prior to Model Building

Statistical QSAR modeling requires the availability of large collections of data. Some examples of known data sources that can be utilized for model QSAR building are provided in Tables 1–3. It is clear that with the recent development and standardization of toxicity data, owing to several data initiatives such as the ToxRefDB [75], DSSTox [76], and NIEHS NTP [77] database projects, we have access to large collections of data, enabling us to model a variety of critically important toxicological endpoints. However, much of this data are not stored in a format suitable for modeling. Thus, chemical structures stored in datasets and databases compiled in Tables 1–3 need to be transformed, i.e., they need to be harmonized and curated, and chemical descriptors should be calculated.

Indeed, molecular modelers typically analyze data generated by other (experimental) researchers. Consequently, when it comes to the experimental data quality, modelers are always at the mercy of data providers. Practically, any cheminformatics study entails the calculation of chemical descriptors that are expected to accurately

Table 1 Publicly available databases of *in vivo* toxicity endpoints

ToxRefDB [75]	Captures multiple toxicological endpoints, including dose–response data from EPA's Office of Pesticide Programs into a relational database using a standardized data field structure and vocabulary
DSSTox dataset [76]	Tumor target site incidence and TD_{50} potencies for 1,354 chemical substances tested in rats and mice, 80 tested in hamsters, 5 tested in dogs, and 27 tested in nonhuman primates
NIEHS/NTP datasets [77]	Data from more than 500 two-year, two species, toxicology, and carcinogenesis studies. The database also contains the results collected on approximately 300 toxicity studies from shorter duration tests and from genetic toxicity studies
FDA adverse liver effects database [78]	Contains generic name of each chemical, SMILES (simply molecular input line entry system) code; includes several modules, i.e., liver enzyme composite module, alkaline phosphatase increased, SGOT increased, SGPT increased, LDH increased, and GGT increased

Table 2 Commercial toxicity databases

Leadscope Toxicity database [79]	Commercial database of over 160,000 chemical structures with toxicity data curated and integrated from multiple sources. Covers acute, multiple dose studies including subchronic liver, carcinogenicity, genetic toxicity, reproductive, and irritation
MDL® Toxicity database [80]	Commercial database of over 150,000 registered toxicological substances. Toxicological profiles consist of acute toxicity, mutagenicity, skin/eye irritation, tumorigenicity and carcinogenicity, and reproductive effects. Reported endpoints include LD_{50}, LC_{50}, and TD_{Lo}
Lhasa Vitic [81]	Approximately 300,000 toxicity data entries for 10,000 compounds. Toxicity endpoints include acute oral toxicity, fish toxicity, carcinogenicity, teratogenicity, and hepatotoxicity

Table 3 Public databases of *in vitro* toxicity endpoints

qHTS cytotoxicity data from NCGC [82]	Concentration–response profiles of 1,408 substances screened for their effects on cell viability are available through PubChem [83] for 13 cell lines
ChEMBLdb [84]	A database of drug-like small molecules abstracted and curated from literature. Bioactivities are represented by binding constants, pharmacology, and ADME/Tox data. ChEMBL assays are available through PubChem
ToxCast™ [85]	Phase I provided 304 unique compounds characterized in over 600 HTS endpoints. Additionally, mapping of these assays to 315 genes and 438 pathways was made publicly available. Phase II will complete screens of additional 700 compounds, and HTS data on nearly 10,000 chemicals will be available through Tox21 collaboration in 2010
ToxNET [86]	A data network covering toxicology, hazardous chemicals, environmental health, and related areas. Managed by the US National Library of Medicine

reflect intricate details of underlying chemical structures. Obviously, any error in the structure translates into either inability to calculate descriptors for erroneous chemical records or into erroneous descriptors; this outcome makes the models developed with such incomplete or inaccurate descriptors either restricted only to a fraction of formally available data or, worse, making the models inaccurate. A recent study [87] showed on average that there are two structural errors per each medicinal chemistry publication, with an overall error rate for compounds indexed in the WOMBAT database [88] as high as 8%. Thus, it is increasingly important to address the issue of chemical and biological data quality that inherently affects the quality of models. In a recent publication [89], we emphasized the importance of this issue for chemical structure analysis and proposed several approaches to curating chemical datasets (cf. approach for Objective 1). Furthermore, recent studies in our group [90] and elsewhere [91] have emphasized the importance of dose–response modeling, including adequate treatment and representation of the raw data as discussed below.

Because models of chemical data are only as good as the data itself, there is a pressing need to develop and systematically employ standard chemical record curation, as illustrated by our recent study of literature assertions concerning drug-induced liver injury (DILI) [92]. The initial dataset of comprised 1,061 molecular structures was processed as follows. First, all inorganic compounds have been removed because our data analysis strategy includes the calculation of molecular descriptors for organic compounds only. Additional compounds were also removed because (1) their corresponding SMILES were impossible to retrieve, or (2) they corresponded to mixtures of compounds. Second, we used the Standardizer module of JChem software [93] to remove all counterions, clean the 2D molecular geometries, and normalize bonds (aromatic, nitro groups, *etc*.) of the remaining compounds. Third, duplicate molecular structures were automatically detected and deleted using the ISIDA/Duplicates program [94], followed by careful manual inspection of the entire dataset. Finally, 951 compounds remained out of the initial 1,061 molecules. It should be pointed out that such laborious steps of data curation are critical to enable accurate QSAR modeling [89, 95].

How significant is the problem of accurate structure representation as it concerns the adequacy and accuracy of cheminformatics models? There appears to be no systematic studies on the subject in the published literature. However, even a few recent reports indicate that this problem should be given very serious attention. For instance, recent benchmarking studies by a large group of collaborators from six laboratories [50, 96] have clearly demonstrated that the type of chemical descriptors has much greater influence on the prediction performances of QSAR models than the nature of the model optimization techniques. Furthermore, in another recent seminal publication [95], the authors clearly pointed out the importance of chemical data curation in the context of QSAR modeling. They have discussed several illustrative examples of incorrect structures generated from either correct or incorrect SMILES using commercial software. They also tried to determine the error rate in several known databases and evaluate the consequences of both random and systematic errors for the prediction performance of QSAR models. Their main conclusions were that small structural errors within a dataset could lead to significant losses in predictive abilities of QSAR models. At the same time, they further demonstrated that manual curation of structural data leads to substantial increase in model predictivity.

Although there are obvious compelling reasons to believe that chemical data curation should be given a lot of attention, it is also obvious that for the most part, the basic steps to curate a dataset of compounds have been either considered trivial or ignored even by experts. For instance, in an effort to improve the quality of publications in the QSAR modeling field, the *Journal of Chemical Information and Modeling* published a special editorial highlighting the requirements to QSAR papers that should be followed should the authors consider publishing their results in the journal [58]; however, no special attention was given to data curation. There have been several recent publications addressing common mistakes and criticizing faulty practices in QSAR modeling field [64, 97, 98]; however, these papers have not explicitly described and discussed the importance of chemical record curation for developing robust QSAR models.

Generally speaking, since the models of chemical data may only be as good as the data itself, there is a pressing need to develop and systematically employ standard chemical record curation protocols that should be helpful in the preprocessing of any chemical dataset. Recently, we have integrated several protocols in a standardized chemical data curation strategy [89] that in our opinion should be followed at the onset of any molecular modeling investigation. The simple, but important, steps for cleaning chemical records in a database include the removal of a fraction of the data that cannot be appropriately handled by conventional cheminformatics techniques, e.g., inorganic and organometallic compounds, counterions, salts, and mixtures; structure validation; ring aromatization; normalization of specific chemotypes; curation of tautomeric forms; and the deletion of duplicates. It is also critical to visualize and manually inspect at least a fraction of chemical data that go into model development to detect structures that for some reasons escaped the automatic curation steps described above.

It is important to realize that most of these structure curation steps do not depend on the level of chemical structure representation, i.e., 2D or 3D, with possible exception of instances when a dataset includes chiral compounds. Obviously, if standard descriptors are calculated from 2D representation of chemical structure,

e.g., by chemical graphs, such as most of molecular connectivity indices [99], then any pair of enantiomers or diastereomers will be formally recognized as duplicates. If specific chiralities for such pairs of compounds are known along with compounds' activities, descriptors taking chirality into account should be used, and all isomers should be retained in the dataset. If, however, chirality information is unavailable, only one compound, usually with the highest (or mean) activity should be retained, and chirality-sensitive descriptors should not be used.

There are different tools available for dataset curation. For example, Molecular Operating Environment (MOE) from CCG [37] includes Database Wash tool. It allows changing molecules' names, adding or removing hydrogen atoms, removing salts and heavy atoms, even if they are covalently connected to the rest of the molecule, and changing or generating the tautomers and protomers (cf. the MOE manual for more details). Various database curation tools are included in ChemAxon [93] as well. If commercial software tools such as MOE are unavailable (notably, the ChemAxon software is free to academic investigators), one can use standard UNIX/LINUX tools to perform some of the dataset cleaning tasks. It is important to have some freely available molecular format converters such as OpenBabel [100], or MolConverter from ChemAxon [93] (Fig. 2).

It is more difficult to spot the errors in biological data since there are no obvious technical approaches similar to chemical record curation that can be used in this case. However, rigorously derived QSAR models could be indeed used to identify compounds for which predictions consistently disagree with experimental observations

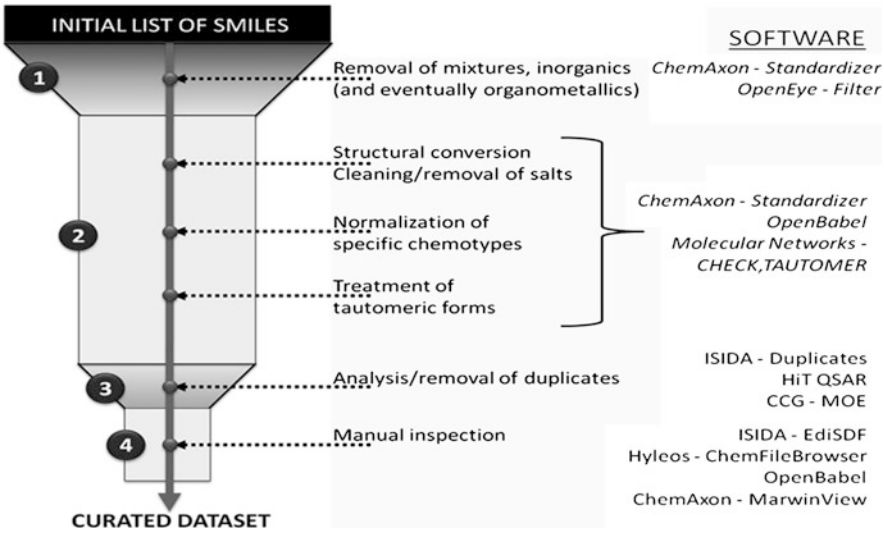


Fig. 2 Workflow for chemical data curation. Major elements of the data curation workflow are discussed in more detail elsewhere [89]. This protocol is enabled by accessible software tools; most of them are publicly available and free-for-academic-use (from ChemAxon [93], OpenEye [101], OpenBabel [100], ISIDA [94], HiT QSAR [102], Hyleos [103]), but some are commercial (from Molecular Networks [104], CCG [37], and CambridgeSoft [105])

and that are likely to be annotated with erroneous biological testing results. Our recent studies provide specific examples demonstrating that the use of cheminformatics approaches even could help spotting gaps or errors in biological annotations of toxic compounds [89, 92].

To conclude this section, the most important steps of dataset cleaning that we described in our recent paper [89] include the removal of inorganics, organometallics, counterions, and mixtures; structural cleaning, ring aromatization, normalization of specific chemotypes, and curation of tautomeric forms; deletion of duplicates; and manual checking of the structures and biological activities. We have formulated the following general rules for chemical data treatment and curation:

- It is risky to calculate chemical descriptors directly from SMILES. It is preferable to compute descriptors (integral, fragments, *etc*.) from curated 2D (or 3D if necessary) chemical structures where all chemotypes are strictly normalized.
- Structural comparison across available databases may facilitate the detection of incorrect structures.
- Even small differences in functional group representations could cause significant errors in models.
- Searching for structure-based duplicates and their removal is one of the mandatory steps in QSAR analysis. Such searches based on chemical name or CAS number only are both incomplete and inefficient.
- Because of the large number of experimental data sources, the search for additional information about investigated property in all available sources is desired to extract valuable knowledge and to compare the data to detect activity cliffs and identify diverse sources of errors.
- Nothing can replace hands-on participation in the process since some errors obvious to a human are still not obvious for computers. After finishing all the mentioned steps, structures and activities should be checked once again manually.

Based on our experience, we suggest that there is a clear need for an additional principle that should be added to the five OECD principles for QSAR model development and validation [64, 65] described above, and this principle should address the need for data curation *before* the model development is initiated. We suggest that this additional principle could be formulated as follows: "To ensure the consideration of (Q)SAR models for regulatory purposes, the models must be trained and validated on chemical datasets that have been thoroughly curated with respect to both chemical structure and associated target property values."

QSAR Modeling of Chemical Toxicity: Examples of Application Studies

As we briefly stated above, most researchers typically apply only one or few independent modeling techniques; however, there is no single method that consistently outperforms others. Our laboratory has consistently advocated for using a

combinatorial QSAR ("Combi-QSAR") methodology [47, 48], which systematically explores various pairwise combinations of computational model optimization methods and chemical descriptors and derives the best models that perform adequately not only in the training set but also in the external validation sets. Furthermore, more recently, we have begun to explore hybrid QSAR methodologies where short-term bioassay results are explored and exploited as additional biological descriptors of chemicals along with traditional chemical descriptors. Our own studies support the use of both combi-QSAR [50] and hybrid models [106, 107] for accurate prediction of chemical toxicity.

Collaborative and Consensus Modeling of Aquatic Toxicity

We shall discuss the results of a recent important study of aquatic toxicity [50]. In our opinion, this particular study may serve as a useful example to illustrate the complexity and power of modern QSAR modeling approaches and highlight the importance of collaborative and consensus model development. Similar views have been recently expressed and realized by a large group of European scientists collaborating on the OpenTOX project [108].

The combinational QSAR modeling approach has been applied to a diverse series of organic compounds tested for aquatic toxicity in *Tetrahymena pyriformis* in the same laboratory over nearly a decade [109–117]. The unique aspect of this research was that it was conducted in collaboration between six academic groups, specializing in cheminformatics and computational toxicology. The common goals for our virtual collaboration were to explore the relative strengths of various QSAR approaches in their ability to develop robust and externally predictive models of this particular toxicity endpoint. We have endeavored to develop the most statistically robust, validated, and *externally* predictive QSAR models of aquatic toxicity. The members of our collaboration included scientists from the University of North Carolina at Chapel Hill (UNC) in the United States, University of Louis Pasteur (ULP) in France, University of Insubria (UI) in Italy, University of Kalmar (UK) in Sweden, Virtual Computational Chemistry Laboratory (VCCLAB) in Germany, and University of British Columbia (UBC) in Canada. Each group relied on its own QSAR modeling approaches to develop toxicity models using the same modeling set, and we agreed to evaluate the realistic model performance using the same external validation set(s).

The *T. pyriformis* toxicity dataset used in this study was compiled from several publications of the Schultz group as well as from data available at the Tetratox database website (http://www.vet.utk.edu/TETRATOX/). After deleting duplicates as well as several compounds with conflicting test results and correcting several chemical structures in the original data sources, our final dataset included 983 unique compounds. The dataset was randomly divided into two parts: (1) the modeling set of 644 compounds and (2) the validation set including 339 compounds. The former set was used for model development by each participating

group, and the latter set was used to estimate the external prediction power of each model as a universal metric of model performance. In addition, when this project was already well under way, a new dataset had become available from the most recent publication by the Schultz group [118]. It provided us with an additional *external* set to evaluate the predictive power and reliability of all QSAR models. Among compounds reported in [118], 110 were unique, i.e., not present among the original set of 983 compounds; thus, these 110 compounds formed the second independent validation set for our study.

Universal Statistical Figures of Merit for All Models

Different groups have employed different techniques and (sometimes) different statistical parameters to evaluate the performance of models developed independently for the modeling set (described below). To harmonize the results of this study, the same standard parameters were chosen to describe each model's performance as applied to the modeling and external test set predictions. Thus, we have employed Q^2_{abs} (squared leave-one-out cross-validation correlation coefficient) for the modeling set, R^2_{abs} (frequently described as coefficient of determination) for the external validation sets, and MAE (mean absolute error) for the linear correlation between predicted (Y_{pred}) and experimental (Y_{exp}) data (here, $Y = p\text{IGC}_{50}$); these parameters are defined as follows:

$$Q^2_{\text{abs}} = 1 - \sum_Y \left(Y_{\text{exp}} - Y_{LOO}\right)^2 \Big/ \sum_Y \left(Y_{\text{exp}} - \langle Y \rangle_{\text{exp}}\right)^2, \tag{1}$$

$$R^2_{\text{abs}} = 1 - \sum_Y \left(Y_{\text{exp}} - Y_{\text{pred}}\right)^2 \Big/ \sum_Y \left(Y_{\text{exp}} - \langle Y \rangle_{\text{exp}}\right)^2, \tag{2}$$

$$\text{MAE} = \sum_Y \left|Y - Y_{\text{pred}}\right| / n. \tag{3}$$

Many other statistical characteristics can be used to evaluate model performance; however, we restricted ourselves to these three parameters that provide minimal but sufficient information concerning any model's ability to reproduce both the trends in experimental data for the test sets as well as mean accuracy of predicting all experimental values. The models were considered acceptable if R^2_{abs} exceeded 0.5.

Consensus QSAR Models of Aquatic Toxicity: Comparison Between Methods and Models

The objective of this study from methodological prospective was to explore the suitability of different QSAR modeling tools for the analysis of a dataset with an important toxicological endpoint. Typically, such datasets are analyzed with one (or several) modeling technique, with a great emphasis on the (high value of) statistical parameters of the training set models. In this study, we went well beyond the modeling studies reported in the original publications in several respects. First, we have compiled all reported data on chemical toxicity against *T. pyriformis* in a single large dataset and attempted to develop global QSAR models for the entire set. Second, we have employed multiple QSAR modeling techniques, thanks to the engagement of six collaborating groups. Third, we have focused on defining model performance criteria not only using training set data but most importantly using external validation sets that were not used in model development in *any* way (unlike any common *cross*-validation procedure) [119]. This focus afforded us the opportunity to evaluate and compare all models using simple and objective universal criteria of *external* predictive accuracy, which in our opinion is the most important single figure of merit for a QSAR model that is of practical significance for experimental toxicologists. Fourth, we have explored the significance of applicability domains and the power of consensus modeling in maximizing the accuracy of external predictivity of our models.

We believe that results of our analysis lend a strong support for our strategy. Indeed, all models performed quite well for the training set with even the lowest Q^2_{abs} among them as high as 0.72. However, there was much greater variation between these models when looking at their (universal and objective) performance criteria as applied to the validation sets.

Of 15 QSAR approaches used in the study, nine implemented method-specific applicability domains. Models that did not define the AD showed a reduced predictive accuracy for the validation set II even though they yielded reasonable results for the validation set I. On average, the use of applicability domains improved the performance of individual models although the improvement came at the expense of the lower chemistry space coverage.

For the most part, all models succeeded in achieving reasonable accuracy of external prediction especially when using the AD. It then appeared natural to bring all models together to explore the power of *consensus prediction*. Thus, the *consensus model* was constructed by averaging all available predicted values, taking into account the applicability domain of each individual model. In this case, we could use only nine of 15 models that had the AD defined. Since each model had its unique way of defining the AD, each external compound could be found within the AD of anywhere between one and nine models, so for averaging, we only used models covering the compound. The advantage of this data treatment is that the overall coverage of the prediction is still high because it was rare to have an external compound outside of the ADs of all available models. The results showed that the prediction accuracy for both the modeling set and the validation sets was the best compared to any individual model. The same observation could be

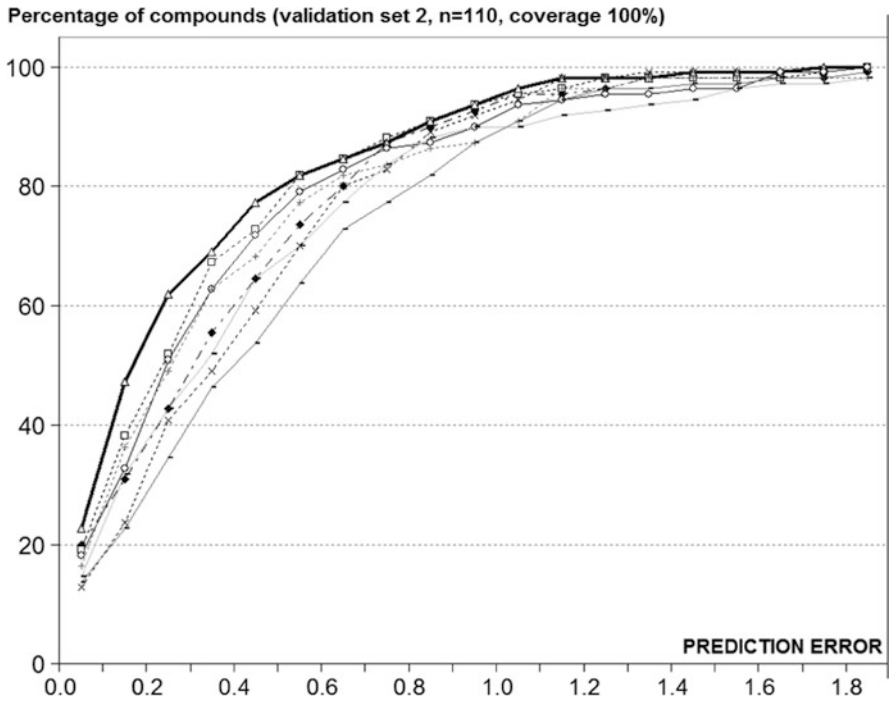


Fig. 3 Percentage of compounds for validation set II with full coverage (100%) *vs*. the prediction errors obtained with individual models and the consensus model

made for the correlation coefficient R^2_{abs}. The coverage of this consensus model II was 100% for all three datasets. This observation suggests that consensus models afford both high space coverage and high accuracy of prediction. Figure 3 illustrates the power of the consensus model as compared to individual models when applied to the prediction of an independent external set. The plot clearly demonstrates that the consensus model always allows to predict the largest number of compounds at a given prediction error, which implies that the consensus model outperforms any individual model in its external prediction power.

In summary, this study presents an example of a fruitful international collaboration between researchers that use different techniques and approaches but share general principles of QSAR model development and validation. Significantly, we did not make any assumptions about the purported mechanisms of aquatic toxicity yet nor were able to develop statistically significant models for all experimentally tested compounds. In this regard, it is relevant to cite an opinion expressed in an earlier publication by Schultz that "models that accurately predict acute toxicity without first identifying toxic mechanisms are highly desirable" [117]. However, the most significant single result of our studies is the demonstrated superior performance of the *consensus modeling* approach when all models are used concurrently and predictions from individual models are averaged. We have shown that both the predictive accuracy and coverage of the final consensus QSAR models

were superior as compared to these parameters for individual models. The consensus models appeared robust in terms of being insensitive to both incorporating individual models with low prediction accuracy and the inclusion or exclusion of the AD. Another important result of this study is the power of addressing complex problems in QSAR modeling by forming a virtual collaboration of independent research groups, leading to the formulation and empirical testing of *best modeling practices*. This latter endeavor is especially critical in light of the growing interest of regulatory agencies to developing most reliable and predictive models for environmental risk assessment [120] and placing such models in the public domain.

Quantitative Structure: In Vitro–In Vivo Relationships

To stress a broad appeal of the conventional QSAR approach, it should be made clear that from the statistical viewpoint, QSAR modeling is a special case of general statistical data mining and data modeling where the data are formatted to represent objects described by multiple descriptors and the robust correlation between descriptors and a target property (e.g., chemical toxicity *in vivo*) is sought. In several computational toxicology studies, additional physicochemical properties, such as water partition coefficient (log *P*) [121], water solubility [122], and melting point [123] were used successfully to augment computed chemical descriptors and improve the predictive power of QSAR models. These studies suggest that using experimental results as descriptors in QSAR modeling could prove beneficial. The currently available and rapidly growing high throughput screening (HTS) data for large and diverse chemical libraries make it possible to extend the scope of the conventional QSAR in toxicity studies by using *in vitro* testing results as additional biological descriptors. In several recent studies, the correlations between various *in vitro* and *in vivo* toxicity testing results were reported [124–127]. However, the earlier reported correlations did not take into account any information about chemical features of underlying compounds. Recently, we hypothesized that the accuracy of models predicting chemical toxicity *in vivo* could improve if we incorporate in the modeling process both chemical descriptors of molecules and the results of short-term biological assays concurrently [106]. We termed this approach quantitative structure *in vitro–in vivo* relationship (QSIIR) modeling. The target properties of QSIIR modeling are still biological activities, such as different *in vivo* toxicity endpoints, but the content and interpretation of "descriptors" and the resulting models will vary. This focus on the prediction of the same target property from different (chemical *and* biological) characteristics of environmental agents affords an opportunity to most fully explore the source-to-outcome continuum of the modern experimental toxicology using cheminformatics approaches. Figure 4 provides visual illustration of the integrated QSIIR approach to *in vivo* toxicity modeling, and the application examples in the following sections of this chapter serve to demonstrate the implementation of this new strategy.

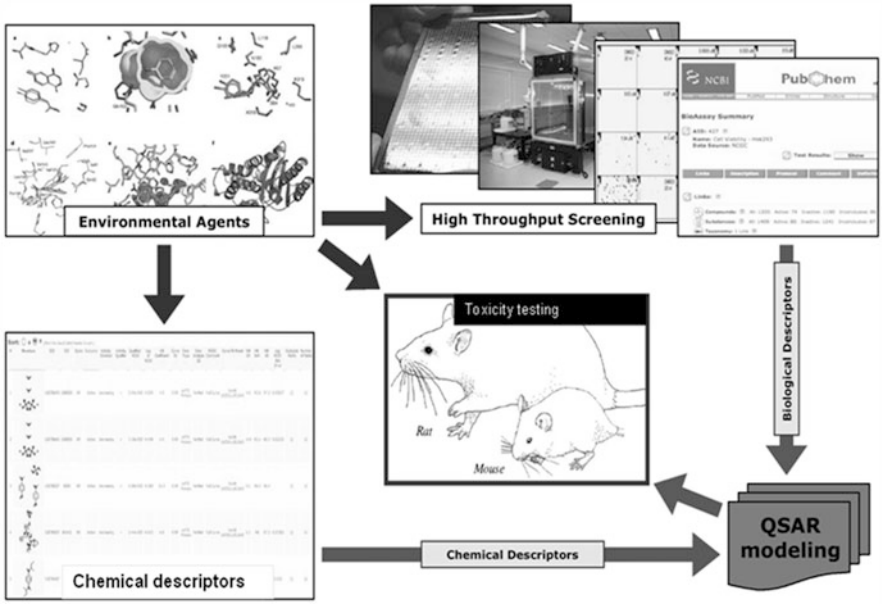


Fig. 4 Combining chemical descriptors and biological screening profiles to create hybrid descriptors for QSIIR modeling of chemical toxicity

QSIIR Modeling of Rodent Carcinogenicity Using "Hybrid" Chemical/ Biological Descriptors

To explore efficient approaches for rapid evaluation of chemical toxicity and human health risk of environmental compounds, NTP in collaboration with the National Center for Chemical Genomics (NCGC) has initiated an HTS Project [128, 129]. The first batch of HTS results for a set of 1,408 compounds tested in six human cell lines were released via PubChem. We have explored these data in terms of their utility for predicting adverse health effects of the environmental agents [106]. Initially, the classification k nearest neighbor (kNN) QSAR modeling method was applied to the HTS data only for the curated dataset of 384 compounds. The resulting models had prediction accuracies for training, test (containing 275 compounds together), and external validation (109 compounds) sets as high as 89%, 71%, and 74%, respectively. We then asked if HTS results could be of value in predicting rodent carcinogenicities. We identified 383 compounds for which data were available from both the Berkeley Carcinogenic Potency Database and NTP-HTS studies. We found that compounds classified by HTS as "actives" in at least one cell line were likely to be rodent carcinogens (sensitivity 77%); however, HTS "inactives" were far less informative (specificity 46%). Using chemical descriptors only, kNN QSAR modeling resulted in 62% overall prediction accuracy for rodent carcinogenicity applied to this dataset. Importantly, the prediction accuracy of the

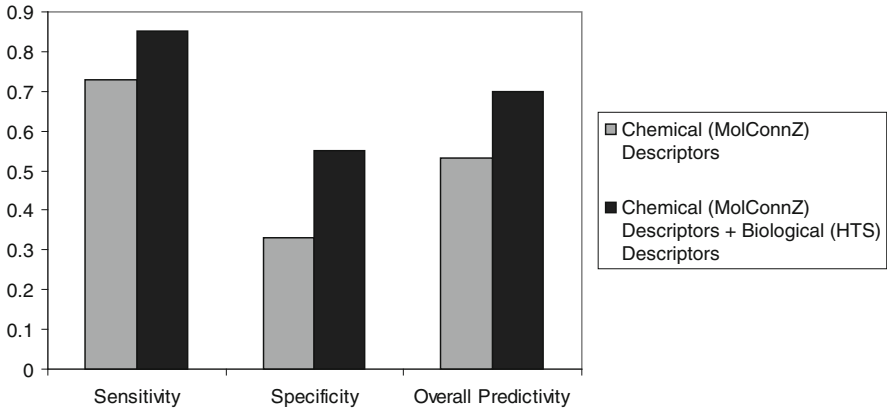


Fig. 5 Comparison of the prediction power of QSTR models of chemical carcinogenicity for the independent validation set using conventional *vs*. hybrid descriptors

model was significantly improved (to 73%) when chemical descriptors were augmented by the HTS data, which were regarded as biological descriptors (Fig. 5).

In a more resent similar study [130], we used the cell viability qHTS data from NCGC as mentioned in the above section for the same 1,408 compounds but in 13 cell lines [131]. Besides the carcinogenicity, we asked if HTS results could be of value in predicting rodent acute toxicity. For this purpose, we have identified 690 of these compounds, for which rodent acute toxicity data (i.e., toxic or nontoxic) were also available. The classification kNN QSAR modeling method was applied to these compounds using either chemical descriptors alone or as a combination of chemical and qHTS biological (hybrid) descriptors as compound features. The external prediction accuracy of models built with chemical descriptors only was 76%. In contrast, the prediction accuracy was significantly improved to 85% when using hybrid descriptors. Both the sensitivities and specificities of hybrid models were clearly better than for conventional QSAR model for predicting the same external compounds. Furthermore, the prediction coverage increased from 76% when using chemical descriptors only to 93% when qHTS biological descriptors were also included. Thus, similar to the previous example, our studies suggest that combining HTS profiles, especially the dose–response qHTS results, with conventional chemical descriptors, could considerably improve the predictive power of QSAR models of rodent toxicity.

Hierarchical QSAR Modeling of Acute Toxicity Based on *In Vitro–In Vivo* Relationships

One potential drawback of models developed by simply combining the chemical and biological profiles is that predictions for external compounds require the results of biological testing. In many cases, we could rely on continuous generation of novel experimental results for environmental chemicals of concern (e.g., through ToxCast™

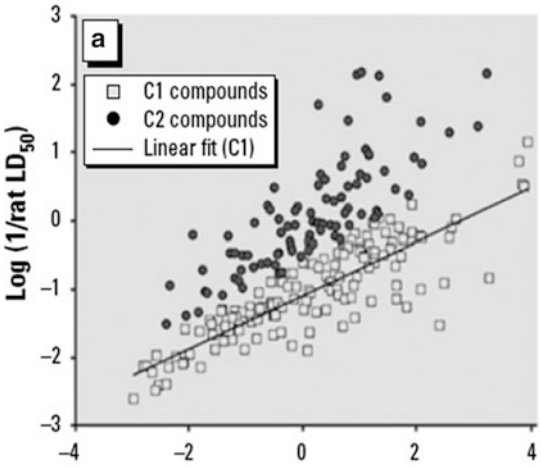


Fig. 6 The identification of the baseline correlation between cytotoxicity (IC_{50}) and rat LD_{50} data

project), which is why the development of hybrid models is highly justified. However, ideally, we may wish to develop models that rely on biological data for the training set but could make prediction for external compounds without having them first tested experimentally. In a recent study [132], we have developed such novel approach to structure *in vitro–in vivo* relationship modeling that ultimately leads to models that could make predictions for external compounds using their chemical descriptors only.

A database containing experimental *in vitro* IC_{50} cytotoxicity values and *in vivo* rodent LD_{50} values for more than 300 chemicals was compiled by the German Center for the Documentation and Validation of Alternative Methods (ZEBET). The application of conventional QSAR modeling approaches to predict mouse or rat acute LD_{50} from chemical descriptors of ZEBET compounds yielded no statistically significant models. Furthermore, analysis of these data showed no general significant correlation between IC_{50} and LD_{50}. However, a linear IC_{50} *vs*. LD_{50} correlation was established for a fraction of compounds (Fig. 6). To capitalize on this observation, a novel two-step modeling approach was developed. First, all chemicals are partitioned into two groups based on the relationship between IC_{50} and LD_{50} values: one group is formed by compounds with linear IC_{50} *vs*. LD_{50} relationship, and another group consists of the remaining compounds. Second, conventional binary classification QSAR models are built to predict the group affiliation based on chemical descriptors only. Third, kNN continuous QSAR models are individually developed for each subclass to predict LD_{50} from chemical descriptors. The workflow illustrating this approach is shown in Fig. 7. All models were extensively validated using special protocols, and an applicability domain was defined for each QSAR model. The prediction for an external compound is realized in two steps as well. First, the classification QSAR model (cf. Fig. 7) is used to assign an external compound to either Class 1 or Class 2.

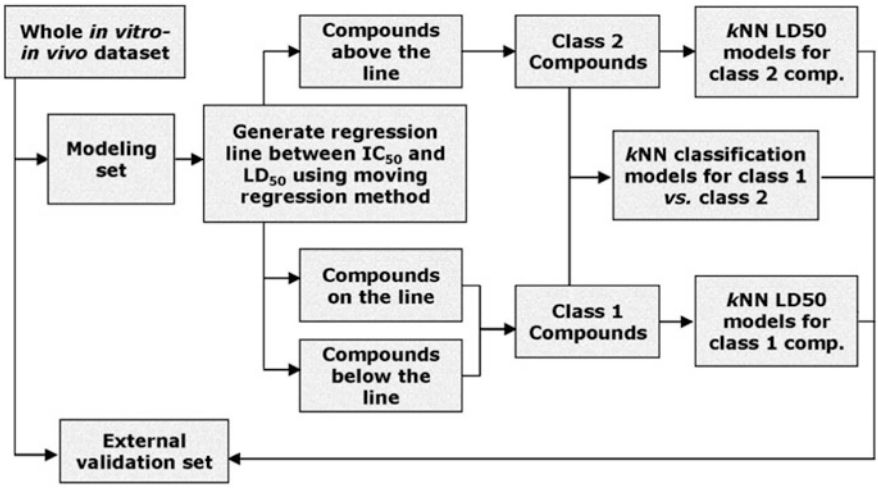


Fig. 7 The two-step hierarchical QSIIR workflow used to develop enhanced rat acute toxicity (LD_{50}) model by using the relationship between LD_{50} and cytotoxicity data (IC_{50}) (cf. Fig. 6) to establish compound classes and kNN QSAR modeling method to build class-specific models

The class-specific continuous QSAR model is used to make a quantitative prediction of LD_{50} values for the same compound. Note that although biological data are used in this approach to train models, only chemical descriptors of an external compound are employed in two different models used sequentially for toxicity prediction. In the end, our models afforded an external prediction accuracy for a dataset of 101 compounds as high as $R^2 = 0.62$ and MAE = 0.42, whereas the performance of *commercial software* TOPKAT for the same 101 compounds was much worse: $R^2 = 0.26$ and MAE = 0.66 [132]. Considering that the TOPKAT LD_{50} training set contains many more compounds (~6,000) than the training set used to develop the two-step model (~200), it is noteworthy that higher prediction accuracy can be achieved using our modeling approach. Thus, similar to examples above, this study provides another illustration of our thesis that the use of *in vitro* data enriches cheminformatics models of chemical toxicity and in fact improves their predictive power.

Making Both Data and Tools Available

We must acknowledge that in the past, the data to develop chemical toxicity models were not readily available. Most of the data were either stored behind the pharmaceutical or chemical company walls or available in print, which required special and tedious effort to convert them in digital format. Not surprisingly, commercial tools for toxicity prediction have prevailed. Most of the popular commercial tools were developed at least a decade ago, including DEREK [133], HazardExpert/CompuDrug [134], Leadscope's Model Applier [79], MultiCASE [135], and TOPKAT

[136]; they rely on internally curated databases that are frequently updated, but the underlying methodologies essentially have remained unchanged. The lack of transparency and commercial nature of these tools precludes their widespread use and validation by computational chemical toxicology community.

However, and as discussed in the first section of this chapter, there are growing collections of data relevant to toxicity assessment in the public domain (cf. Tables 1 and 3), which enable the development of computational tools and models that can be placed in the public domain as well. The majority of free tools are indeed relatively recent and, for the most part, are still under development, including OpenTOX [108], OECD (Q)SAR Application Toolbox [137], EPA T.E.S.T. [138], EPA EPI Suite [139], and REACH CheSAR [140].

As an example of a recent public effort, and fueled by the urgent need to evaluate potential health risks associated with human exposure to the REACH chemicals [141], the Organization for Economic Development and Cooperation (OECD) has funded the development of the (Q)SAR Application Toolbox [137]. The goal of this publicly funded effort is to increase regulatory acceptance of (Q)SAR approaches; the models are "intended to be used by governments, chemical industry and other stakeholders in filling gaps in (eco)toxicity data needed for assessing the hazards of chemicals."

The OpenTOX project [108] is another example of a recent public effort also funded by the European Union. This project (described in a recent publication [142]) is a public joint effort of several European laboratories that collaborate on developing a publicly available computational toxicology framework. The project focuses on the development of interoperable infrastructure integrating publicly available data, data ontologies, and software to enable the reliable environmental chemical risk assessment, especially for the REACH [141] chemicals.

In a recent publication, Ekins and Williams have appealed to the scientific community to make ADME/Tox data free on the web to "facilitate computational model building and assist drug development" [143]. They argued that these data are precompetitive in nature and that their release will help researchers to steer drug discovery projects toward safe clinical candidates. We would add that the problem of chemical safety extends beyond drug candidates toward the environmental compounds as well. Since the development of safe chemicals implies the lack of human and animal toxicity, setting all toxicity data "free on the web" will facilitate the development of better models of environmental toxicity.

The emergence of publicly available tools represents an important new trend supported by national and international agencies, especially in Europe (such as OpenTOX and OECD (Q)SAR projects) that is very likely to continue fueled by the increased availability of data. Another important area in chemical toxicology, which goes hand in hand with providing freely accessible data, is the development of not only freely available but also easily accessible computational tools and models. Of course, there is an obvious question as to whether noncommercial tools will be able to compete with those developed by companies specializing in chemical toxicity prediction. However, a recent study conducted within Pfizer [144] suggests that ADME/Tox QSAR models developed with publicly available

chemical descriptors, and data mining methods were on par in their performance with commercial tools for the same internal datasets.

The most obvious way to facilitate access to both tools and models is to make them available via specialized web portals. Our group has initiated efforts in this direction by developing the publicly available ChemBench web portal (http://chembench.mml.unc.edu) [73]. The system is designed to integrate translational cheminformatics research conducted in our group over a period of more than 15 years and in collaborating laboratories elsewhere. The current ChemBench system consists of four modules: *Dataset*, *Modeling*, *Prediction*, and *My Bench*.

The functionality contained under *Dataset* allows users to upload, store, and standardize chemical structures. MolconnZ and Dragon descriptors, as well as MOE and MACCS keys, are generated for each compound. If the dataset is intended for modeling, the user must include activity data for each compound. The user can either manually or automatically select an external set for validating the modeling process. Additionally, after creating a dataset, the user can access the dataset via the *My Bench* tab to view the chemical structures, examine the distribution of activities, and generate a heat map to check for obvious relationships between global compound similarity and activity. Through *Modeling*, users can select a dataset (either an uploaded dataset or a provided benchmark) and build a related predictor. The user has control over many of the modeling parameters, such as descriptors, modeling algorithms, feature selection parameters, and the internal validation paradigm. The user receives an optional email notification when the modeling is complete, and the predictor can then be accessed from the *My Bench* page. The consensus predictor's performance on each of the compounds in the external validation set and details for each individual model, including r^2, q^2, and the descriptors used, are shown. The *Prediction* page allows users to predict compound activities using one or more of the developed predictors. Several predictors developed within our group, e.g., models of aquatic toxicity discussed above [50] are already available for users. Once the user has selected which predictors to use, they may specify one of the stored datasets to start a prediction calculation or draw a compound and predict its activity interactively. Completed prediction calculations are accessible through *My Bench*. Similar systems are beginning to emerge elsewhere, e.g., the OCHEM project (http://ochem.eu/).

In summary, we believe there is an important emerging trend in computational toxicology toward establishing innovative, robust, and freely available software tools and models for accurate prediction of chemical toxicity. We anticipate that this trend will continue to develop in the near future.

Summary and Prospects

In this chapter, the developing trends in computational chemical toxicology that entail growing availability of data collections (both *in vitro* and *in vivo*), increased rigor of computational tools and models, especially with respect to greater emphasis

on model validation, and the emergence of freely available and accessible tools and models are reviewed. The support by both government and nonprofit organizations for chemical toxicology research includes sponsored development of both publicly available data collections (cf. Tables 1 and 3) as well as computational tools. These recent developments provide new opportunities for developing novel approaches and innovative data processing workflows to improve the quality of chemical toxicity models, increase their use in experimental toxicology research to cut both time and cost of toxicity studies, substantially decrease the use of animals, and promote the use of models in regulatory decision making.

Acknowledgments The author is grateful to members of his laboratory who have been involved in research projects described herein as well as colleagues at EPA and UNC for many fruitful discussions. The author also appreciates the financial support of his laboratory from NIH (grants R01GM066940 and R21GM076059) and EPA (grants RD832720 and RD833825).

References

1. Collins FS, Gray GM, Bucher JR (2008) Toxicology—transforming environmental health protection. Science 319:906–907
2. (2007) Toxicity testing in the 21st century: a vision and a strategy. Committee on Toxicity Testing and Assessment of Environmental Agents, National Research Council, National Academy of Science, The National Academies Press, Washington, DC
3. Shukla SJ, Huang R, Austin CP, Xia M (2010) The future of toxicity testing: a focus on *in vitro* methods using a quantitative high-throughput screening platform. Drug Discov Today 15:997–1007
4. Judson RS, Houck KA, Kavlock RJ, Knudsen TB, Martin MT, Mortensen HM, Reif DM, Rotroff DM, Shah I, Richard AM, Dix DJ (2010) *In vitro* screening of environmental chemicals for targeted testing prioritization: the ToxCast project. Environ Health Perspect 118:485–492
5. Tunkel J, Mayo K, Austin C, Hickerson A, Howard P (2005) Practical considerations on the use of predictive models for regulatory purposes. Environ Sci Technol 39:2188–2199
6. Zvinavashe E, Murk AJ, Rietjens IM (2008) Promises and pitfalls of quantitative structure-activity relationship approaches for predicting metabolism and toxicity. Chem Res Toxicol 21:2229–2236
7. Miller JA, Miller EC (1977) Ultimate chemical carcinogen as reactive mutagenic electrophiles. In: Hiatt HH, Watson JD, Winsten JA (eds) Origin of human cancers. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 605–627
8. Ashby J, Tennant RW (1988) Chemical structure, *Salmonella* mutagenicity and extent of carcinogenicity as indicators of genotoxic carcinogenesis among 222 chemicals tested in rodents by the U.S. NCI/NTP. Mutat Res 204:17–115
9. Ashby J, Tennant RW, Zeiger E, Stasiewicz S (1989) Classification according to chemical structure, mutagenicity to *Salmonella* and level of carcinogenicity of a further 42 chemicals tested for carcinogenicity by the U.S. National Toxicology Program. Mutat Res 223:73–103
10. Ashby J, Tennant RW (1991) Definitive relationships among chemical structure, carcinogenicity and mutagenicity for 301 chemicals tested by the U.S. NTP. Mutat Res 257:229–306
11. Tennant RW, Ashby J (1991) Classification according to chemical structure, mutagenicity to *Salmonella* and level of carcinogenicity of a further 39 chemicals tested for carcinogenicity by the U.S. National Toxicology Program. Mutat Res 257:209–227
12. Votano JR, Parham M, Hall LH, Kier LB, Oloff S, Tropsha A, Xie Q, Tong W (2004) Three new consensus QSAR models for the prediction of Ames genotoxicity. Mutagenesis 19:365–377

13. Valerio LG Jr (2009) *In silico* toxicology for the pharmaceutical sciences. Toxicol Appl Pharmacol 241:356–370
14. Durham SK, Pearl GM (2001) Computational methods to predict drug safety liabilities. Curr Opin Drug Discov Devel 4:110–115
15. Jacobson-Kram D, Contrera JF (2007) Genetic toxicity assessment: employing the best science for human safety evaluation. Part I: Early screening for potential human mutagens. Toxicol Sci 96:16–20
16. Muster W, Breidenbach A, Fischer H, Kirchner S, Müller L, Pahler A (2008) Computational toxicology in drug development. Drug Discov Today 13:303–310
17. Snyder RD (2009) An update on the genotoxicity and carcinogenicity of marketed pharmaceuticals with reference to in *silico* predictivity. Environ Mol Mutagen 50:435–450
18. Bailey AB, Chanderbhan R, Collazo-Braier N, Cheeseman MA, Twaroski ML (2005) The use of structure-activity relationship analysis in the food contact notification program. Regul Toxicol Pharmacol 42:225–235
19. Valerio LG Jr (2008) Tools for evidence-based toxicology: computational-based strategies as a viable modality for decision support in chemical safety evaluation and risk assessment. Hum Exp Toxicol 27:757–760
20. Zvinavashe E, Murk AJ, Rietjens IM (2009) On the number of EINECS compounds that can be covered by (Q)SAR models for acute toxicity. Toxicol Lett 184:67–72
21. Stouch TR, Kenyon JR, Johnson SR, Chen XQ, Doweyko A, Li Y (2003) *In silico* ADME/Tox: why models fail. J Comput Aided Mol Des 17:83–92
22. Lombardo F, Gifford E, Shalaeva MY (2003) *In silico* ADME prediction: data, models, facts and myths. Mini Rev Med Chem 3:861–875
23. Hansch C, Fujita T (1964) r-s-p Analysis. A method for the correlation of biological activity and chemical structure. J Am Chem Soc 86:1616–1626
24. Zheng W, Tropsha A (2000) Novel variable selection quantitative structure-property relationship approach based on the k-nearest-neighbor principle. J Chem Inf Comput Sci 40:185–194
25. Tropsha A (2005) Application of predictive QSAR models to database mining. In: Oprea TI (ed) Cheminformatics in drug discovery. Wiley-VCH, New York, NY, pp 437–455
26. Tropsha A (2003) Recent trends in quantitative structure-activity relationships. In: Abraham D (ed) Burger's medicinal chemistry and drug discovery. Wiley, New York, NY, pp 49–77
27. Tropsha A (2006) Predictive QSAR (quantitative structure activity relationships) modeling. In: Martin YC (ed) Comprehensive medicinal chemistry II. Elsevier, Amsterdam, pp 113–126
28. Papa E, Villa F, Gramatica P (2005) Statistically validated QSARs, based on theoretical descriptors, for modeling aquatic toxicity of organic chemicals in *Pimephales promelas* (fathead minnow). J Chem Inf Model 45:1256–1266
29. Tetko IV (2002) Neural network studies. 4. Introduction to associative neural networks. J Chem Inf Comput Sci 42:717–728
30. Zupan J, Novic M, Gasteiger J (1995) Neural networks with counter-propagation learning-strategy used for modelling. Chemometr Intell Lab Syst 27:175–187
31. Devillers J (1996) Strengths and weaknesses of the back propagation neural network in QSAR and QSPR studies. In: Devillers J (ed) Genetic algorithms in molecular modeling. Academic, London, pp 1–24
32. Engels MFM, Wouters L, Verbeeck R, Vanhoof G (2002) Outlier mining in high throughput screening experiments. J Biomol Screen 7:341–351
33. Schuurmann G, Aptula AO, Kuhne R, Ebert RU (2003) Stepwise discrimination between four modes of toxic action of phenols in the *Tetrahymena pyriformis* assay. Chem Res Toxicol 16:974–987
34. Xue Y, Li H, Ung CY, Yap CW, Chen YZ (2006) Classification of a diverse set of *Tetrahymena pyriformis* toxicity chemical compounds from molecular descriptors by statistical learning methods. Chem Res Toxicol 19:1030–1039
35. Breiman L, Friedman JH, Olshen RA, Stone CJ (1984) Classification and regression trees. Wadsworth & Brooks, Monterey, CA

36. Deconinck E, Hancock T, Coomans D, Massart DL, van der Heyden Y (2005) Classification of drugs in absorption classes using the classification and regression trees (CART) methodology. J Pharm Biomed Anal 39:91–103
37. MOE, Molecular Operating Environment (2005) http://www.chemcomp.com/fdept/prodinfo.htm#Cheminformatics. Accessed Jan 2005
38. Put R, Perrin C, Questier F, Coomans D, Massart DL, van der Heyden Y (2003) Classification and regression tree analysis for molecular descriptor selection and retention prediction in chromatographic quantitative structure-retention relationship studies. J Chromatogr A 988:261–276
39. Breiman L (2001) Random forests. Machine Learn 45:5–32
40. Svetnik V, Liaw A, Tong C, Culberson JC, Sheridan RP, Feuston BP (2003) Random forest: a classification and regression tool for compound classification and QSAR modeling. J Chem Inf Comput Sci 43:1947–1958
41. Put R, Xu QS, Massart DL, van der Heyden Y (2004) Multivariate adaptive regression splines (MARS) in chromatographic quantitative structure-retention relationship studies. J Chromatogr A 1055:11–19
42. Friedman JH (1991) Multivariate adaptive regression splines. Ann Stat 19:1–67
43. Vapnik VN (1995) The nature of statistical learning theory. Springer, New York, NY
44. Aires-de-Sousa J, Gasteiger J (2005) Prediction of enantiomeric excess in a combinatorial library of catalytic enantioselective reactions. J Comb Chem 7:298–301
45. Oloff S, Mailman RB, Tropsha A (2005) Application of validated QSAR models of D1 dopaminergic antagonists for database mining. J Med Chem 48:7322–7332
46. Chohan KK, Paine SW, Waters NJ (2006) Quantitative structure activity relationships in drug metabolism. Curr Top Med Chem 6:1569–1578
47. de Cerqueira LP, Golbraikh A, Oloff S, Xiao Y, Tropsha A (2006) Combinatorial QSAR modeling of P-glycoprotein substrates. J Chem Inf Model 46:1245–1254
48. Kovatcheva A, Golbraikh A, Oloff S, Xiao YD, Zheng W, Wolschann P, Buchbauer G, Tropsha A (2004) Combinatorial QSAR of ambergris fragrance compounds. J Chem Inf Comput Sci 44:582–595
49. Wang XS, Tang H, Golbraikh A, Tropsha A (2008) Combinatorial QSAR modeling of specificity and subtype selectivity of ligands binding to serotonin receptors 5HT1E and 5HT1F. J Chem Inf Model 48:997–1013
50. Zhu H, Tropsha A, Fourches D, Varnek A, Papa E, Gramatica P, Oberg T, Dao P, Cherkasov A, Tetko IV (2008) Combinatorial QSAR modeling of chemical toxicants tested against *Tetrahymena pyriformis*. J Chem Inf Model 48:766–784
51. Tropsha A, Gramatica P, Gombar VK (2003) The importance of being earnest: validation is the absolute essential for successful application and interpretation of QSPR models. Quant Struct Act Relat Comb Sci 22:69–77
52. Golbraikh A, Tropsha A (2002) Predictive QSAR modeling based on diversity sampling of experimental datasets for the training and test set selection. J Comput Aided Mol Des 16: 357–369
53. Norinder U (1996) Single and domain made variable selection in 3D QSAR applications. J Chemomet 10:95–105
54. Zefirov NS, Palyulin VA (2001) QSAR for boiling points of "small" sulfides. Are the "high-quality structure-property-activity regressions" the real high quality QSAR models? J Chem Inf Comput Sci 41:1022–1027
55. Kubinyi H, Hamprecht FA, Mietzner T (1998) Three-dimensional quantitative similarity-activity relationships (3D QSiAR) from SEAL similarity matrices. J Med Chem 41:2553–2564
56. Novellino E, Fattorusso C, Greco G (1995) Use of comparative molecular field analysis and cluster analysis in series design. Pharm Acta Helv 70:149–154
57. Golbraikh A, Tropsha A (2002) Beware of q2! J Mol Graph Model 20:269–276
58. Jorgensen WL (2006) QSAR/QSPR and proprietary data. J Chem Inf Model 46:937

59. Tropsha A, Cho SJ (1998) Cross-validated R2-guided region selection for CoMFA studies. In: Kubinyi H, Folkers G, Martin YC (eds) 3D QSAR in drug design. Kluwer Academic, Dordrecht, pp 57–69
60. Golbraikh A, Shen M, Xiao Z, Xiao YD, Lee KH, Tropsha A (2003) Rational selection of training and test sets for the development of validated QSAR models. J Comput Aided Mol Des 17:241–253
61. Moss GP, Cronin MTD (2002) Quantitative structure-permeability relationships for percutaneous absorption: re-analysis of steroid data. Int J Pharm 238:105–109
62. Estrada E, Patlewicz G (2004) On the usefulness of graph-theoretic descriptors in predicting theoretical parameters. Phototoxicity of polycyclic aromatic hydrocarbons (PAHs). Croat Chem Acta 77:203–211
63. Leo AJ, Hansch C (1999) Role of hydrophobic effects in mechanistic QSAR. Perspect Drug Discov Des 17:1–25
64. Dearden JC, Cronin MT, Kaiser KL (2009) How not to develop a quantitative structure-activity or structure-property relationship (QSAR/QSPR). SAR QSAR Environ Res 20: 241–266
65. QSAR Expert Group (2004) The Report from the Expert Group on (Quantitative) structure-activity relationships [(Q)SARs] on the principles for the validation of (Q)SARs. OECD (Organisation for Economic Co-operation and Development) Paris 49: 206
66. Pavan M, Netzeva TI, Worth AP (2006) Validation of a QSAR model for acute toxicity. SAR QSAR Environ Res 17:147–171
67. Vracko M, Bandelj V, Barbieri P, Benfenati E, Chaudhry Q, Cronin M, Devillers J, Gallegos A, Gini G, Gramatica P, Helma C, Mazzatorta P, Neagu D, Netzeva T, Pavan M, Patlewicz G, Randić M, Tsakovska I, Worth AP (2006) Validation of counter propagation neural network models for predictive toxicology according to the OECD principles: a case study. SAR QSAR Environ Res 17:265–284
68. Saliner AG, Netzeva TI, Worth AP (2006) Prediction of estrogenicity: validation of a classification model. SAR QSAR Environ Res 17:195–223
69. Roberts DW, Aptula AO, Patlewicz G (2006) Mechanistic applicability domains for non-animal based prediction of toxicological endpoints. QSAR analysis of the schiff base applicability domain for skin sensitization. Chem Res Toxicol 19:1228–1233
70. Zhang S, Golbraikh A, Tropsha A (2006) Development of quantitative structure-binding affinity relationship models based on novel geometrical chemical descriptors of the protein-ligand interfaces. J Med Chem 49:2713–2724
71. Tropsha A (2010) Best practices for QSAR model development, validation, and exploitation. Mol Inf 29:476–488
72. Tropsha A, Golbraikh A (2007) Predictive QSAR modeling workflow, model applicability domains, and virtual screening. Curr Pharm Des 13:3494–3504
73. Walker T, Grulke CM, Pozefsky D, Tropsha A (2010) Chembench: a cheminformatics workbench. Bioinformatics 26:3000–3001
74. Golbraikh A, Tropsha A (2002) Predictive QSAR modeling based on diversity sampling of experimental datasets for the training and test set selection. Mol Divers 5:231–243
75. ToxRefDB (2010) http://www.epa.gov/NCCT/toxrefdb. Accessed Jan 2010
76. DSSTox (2010) http://www.epa.gov/comptox/dsstox/. Accessed Jan 2010
77. ToxRefDB (2010) http://actor.epa.gov/toxrefdb/faces/Home.jsp. Accessed Jan 2010
78. FDA Liver Side Effect (2010) http://www.fda.gov/AboutFDA/CentersOffices/CDER/ucm092203.htm. Accessed Jan 2010
79. Leadscope (2010) http://www.leadscope.com/product_info.php?products_id=78. Accessed Jan 2010
80. MDL® Toxicity (2010) www.symyx.com/products/pdfs/toxds.pdf. Accessed Jan 2010
81. Lhasa Vitic (2010) http://www.lhasalimited.org/vitic_nexus/. Accessed Jan 2010
82. NCGC, National Cencer of Chemical Genomics (2010) http://www.ncgc.nih.gov. Accessed Jan 2010

83. PubChem (2008) http://pubchem.ncbi.nlm.nih.gov/. Accessed Jan 2008
84. ChEMBLdb (2010) http://www.ebi.ac.uk/chembldb/index.php. Accessed Jan 2010
85. ToxCast™ (2010) http://www.epa.gov/comptox/toxcast/. Accessed Jan 2010
86. ToxNET (2010) http://www.toxnet.nlm.nih.gov. Accessed Jan 2010
87. Olah M, Mracec M, Ostopovici L, Rad R, Bora A, Hadaruga N, Olah I, Banda M, Simon Z, Mracec M, Oprea TI (2005) WOMBAT: world of molecular bioactivity. In: Oprea TI (ed) Chemoinformatics in drug discovery, Chap 9. Wiley-VCH, New York, NY, pp 223–239
88. Olah M, Rad R, Ostopovici L, Bora A, Hadaruga N, Hadaruga D, Moldovan R, Fulias A, Mracec M, Oprea TI (2007) WOMBAT and WOMBAT-PK: bioactivity databases for lead and drug discovery. In: Schreiber SL, Kapoor TM, Weiss G (eds) Chemical biology: from small molecules to systems biology and drug design, Vol 2. Wiley-VCH, Weinheim, pp 760–786
89. Fourches D, Muratov E, Tropsha A (2010) Trust, but verify: on the importance of chemical structure curation in cheminformatics and QSAR modeling research. J Chem Inf Model 50:1189–1204
90. Sedykh A, Zhu H, Tang H, Zhang L, Richard A, Rusyn I, Tropsha A (2010) Use of *in vitro* HTS-derived concentration-response data as biological descriptors improves the accuracy of QSAR models of *in vivo* toxicity. Environ Health Perspect 119:364–370
91. Parham F, Austin C, Southall N, Huang R, Tice R, Portier C (2009) Dose-response modeling of high-throughput screening data. J Biomol Screen 14:1216–1227
92. Fourches D, Barnes JC, Day NC, Bradley P, Reed JZ, Tropsha A (2010) Cheminformatics analysis of assertions mined from literature that describe drug-induced liver injury in different species. Chem Res Toxicol 23:171–183
93. ChemAxon (2010) ChemAxon JChem. http://www.chemaxon.com. Accessed Jan 2010
94. ISIDA Software (2010) Louis Pasteur University Strasbourg, Laboratoire d'Infochimie, France. http://infochim.u-strasbg.fr. Accessed Jan 2010
95. Young D, Martin D, Venkatapathy R, Harten P (2008) Are the chemical structures in your QSAR correct? QSAR Comb Sci 27:1337–1345
96. Tetko IV, Sushko I, Pandey AK, Zhu H, Tropsha A, Papa E, Oberg T, Todeschini R, Fourches D, Varnek A (2008) Critical assessment of QSAR models of environmental toxicity against *Tetrahymena pyriformis*: focusing on applicability domain and overfitting by variable selection. J Chem Inf Model 48:1733–1746
97. Maggiora GM (2006) On outliers and activity cliffs—why QSAR often disappoints. J Chem Inf Model 46:1535
98. Johnson SR (2008) The trouble with QSAR (or how I learned to stop worrying and embrace fallacy). J Chem Inf Model 48:25–26
99. Kier LB, Hall LH (1976) Molecular connectivity in chemistry and research. Academic, New York, NY
100. OpenBabel (2010) The OpenSource chemistry toolbox. http://www.Openbabel.org. Accessed Jan 2010
101. OpenEye Scientific Software (2010) http://www.eyesopen.com/products/applications/filter.html. Accessed Jan 2010
102. Kuz'min VE, Artemenko AG, Muratov EN (2008) Hierarchical QSAR technology on the base of simplex representation of molecular structure. J Comp Aid Mol Des 22:403–421
103. Hyleos (2010) http://www.hyleos.net/. Accessed Jan 2010
104. Molecular Networks GmbH (2010) http://www.molecular-networks.com/products. Accessed Jan 2010
105. CambridgeSoft (2009) http://www.cambridgesoft.com/. Accessed Jan 2009
106. Zhu H, Rusyn I, Richard A, Tropsha A (2008) Use of cell viability assay data improves the prediction accuracy of conventional quantitative structure-activity relationship models of animal carcinogenicity. Environ Health Perspect 116:506–513

107. Zhu H, Ye L, Richard A, Golbraikh A, Wright FA, Rusyn I, Tropsha A (2009) A novel two-step hierarchical quantitative structure-activity relationship modeling work flow for predicting acute toxicity of chemicals in rodents. Environ Health Perspect 117:1257–1264
108. OpenTOX (2010) http://www.opentox.org. Accessed Jan 2010
109. Aptula AO, Roberts DW, Cronin MTD, Schultz TW (2005) Chemistry-toxicity relationships for the effects of di- and trihydroxybenzenes to *Tetrahymena pyriformis*. Chem Res Toxicol 18:844–854
110. Netzeva TI, Schultz TW (2005) QSARs for the aquatic toxicity of aromatic aldehydes from *Tetrahymena* data. Chemosphere 61:1632–1643
111. Schultz TW, Sinks GD, Miller LA (2001) Population growth impairment of sulfur-containing compounds to *Tetrahymena pyriformis*. Environ Toxicol 16:543–549
112. Schultz TW, Cronin MT, Netzeva TI, Aptula AO (2002) Structure-toxicity relationships for aliphatic chemicals evaluated with *Tetrahymena pyriformis*. Chem Res Toxicol 15: 1602–1609
113. Schultz TW, Netzeva TI, Cronin MT (2003) Selection of data sets for QSARs: Analyses of *Tetrahymena* toxicity from aromatic compounds. SAR QSAR Environ Res 14:59–81
114. Schultz TW, Netzeva TI (2004) Development and evaluation of QSARs for ecotoxic endpoints: The benzene response-surface model for *Tetrahymena* toxicity. In: Cronin MTD, Livingstone DJ (eds) Modeling environmental fate and toxicity. CRC, Boca Raton, FL, pp 265–284
115. Schultz TW, Netzeva TI, Roberts DW, Cronin MT (2005) Structure-toxicity relationships for the effects to Tetrahymena pyriformis of aliphatic, carbonyl-containing, α,β-unsaturated chemicals. Chem Res Toxicol 18:330–341
116. Schultz TW, Yarbrough JW, Woldemeskel M (2005) Toxicity to *Tetrahymena* and abiotic thiol reactivity of aromatic isothiocyanates. Cell Biol Toxicol 21:181–189
117. Schultz TW (1999) Structure-toxicity relationships for benzenes evaluated with *Tetrahymena pyriformis*. Chem Res Toxicol 12:1262–1267
118. Schultz TW, Hewitt M, Netzeva TI, Cronin MTD (2007) Assessing applicability domains of toxicological QSARs: definition, confidence in predicted values, and the role of mechanisms of action. QSAR Comb Sci 26:238–254
119. Gramatica P (2007) Principles of QSAR models validation: internal and external. QSAR Comb Sci 26:694–701
120. Yang C, Richard AM, Cross KP (2006) The art of data mining the minefields of toxicity databases to link chemistry to biology. Curr Comput Aided Drug Des 2:135–150
121. Klopman G, Zhu H, Ecker G, Chiba P (2003) MCASE study of the multidrug resistance reversal activity of propafenone analogs. J Comput Aided Mol Des 17:291–297
122. Stoner CL, Gifford E, Stankovic C, Lepsy CS, Brodfuehrer J, Prasad JV, Surendran N (2004) Implementation of an ADME enabling selection and visualization tool for drug discovery. J Pharm Sci 93:1131–1141
123. Mayer P, Reichenberg F (2006) Can highly hydrophobic organic substances cause aquatic baseline toxicity and can they contribute to mixture toxicity? Environ Toxicol Chem 25: 2639–2644
124. Forsby A, Blaauboer B (2007) Integration of *in vitro* neurotoxicity data with biokinetic modelling for the estimation of *in vivo* neurotoxicity. Hum Exp Toxicol 26:333–338
125. Schirmer K, Tanneberger K, Kramer NI, Volker D, Scholz S, Hafner C, Lee LC, Bols NC, Hermens JL (2008) Developing a list of reference chemicals for testing alternatives to whole fish toxicity tests. Aquat Toxicol 90:128–137
126. Piersma AH, Janer G, Wolterink G, Bessems JG, Hakkert BC, Slob W (2008) Quantitative extrapolation of *in vitro* whole embryo culture embryotoxicity data to developmental toxicity *in vivo* using the benchmark dose approach. Toxicol Sci 101:91–100
127. Sjostrom M, Kolman A, Clemedson C, Clothier R (2008) Estimation of human blood LC_{50} values for use in modeling of *in vitro-in vivo* data of the ACuteTox project. Toxicol In Vitro 22:1405–1411

128. Inglese J, Auld DS, Jadhav A, Johnson RL, Simeonov A, Yasgar A, Zheng W, Austin CP (2006) Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries. Proc Natl Acad Sci USA 103: 11473–11478
129. Thomas CJ, Auld DS, Huang R, Huang W, Jadhav A, Johnson RL, Leister W, Maloney DJ, Marugan JJ, Michael S, Simeonov A, Southall N, Xia M, Zheng W, Inglese J, Austin CP (2009) The pilot phase of the NIH Chemical Genomics Center. Curr Top Med Chem 9: 1181–1193
130. Sedykh A, Zhu H, Tang H, Zhang L, Rusyn I, Richard A, Rusyn I, Tropsha A (2011) The use of dose-response qHTS data as biological descriptors improves the prediction accuracy of QSAR models of acute rat toxicity. Environ Health Perspect 119:364–370
131. Xia M, Huang R, Witt KL, Southall N, Fostel J, Cho MH, Jadhav A, Smith CS, Inglese J, Portier CJ, Tice RR, Austin CP (2008) Compound cytotoxicity profiling using quantitative high-throughput screening. Environ Health Perspect 116:284–291
132. Zhu H, Martin TM, Ye L, Sedykh A, Young DM, Tropsha A (2009) Quantitative structure-activity relationship modeling of rat acute toxicity by oral exposure. Chem Res Toxicol 22:1913–1921
133. DEREK (2010) http://www.lhasalimited.org/index.php/derek/. Accessed Jan 2010
134. CompuDrug (2010) http://www.compudrug.com. Accessed Jan 2010
135. MultiCASE (2010) http://www.multicase.com. Accessed Jan 2010
136. TOPKAT (2010) http://www.accelrys.com/mini/toxicology/predictive-functionality.html. Accessed Jan 2010
137. OECD (Q)SAR Project (2010) http://www.oecd.org/document/23/0,3343,en_2649_34379_33957015_1_1_1_1,00.html. Accessed Jan 2010
138. EPA QSAR (2010) http://www.epa.gov/nrmrl/std/cppb/qsar/. Accessed Jan 2010
139. EPA EPI (2010) http://www.epa.gov/oppt/exposure/pubs/episuite.htm. Accessed Jan 2010
140. REACH CheSAR (2010) http://www.reach-compliance.eu/english/compliance/CSA/CHESAR_CSA_CSR_Tool.html. Accessed Jan 2010
141. REACH Regulation (2010) http://ec.europa.eu/environment/chemicals/reach/reach_intro.htm. Accessed Jan 2010
142. Hardy B, Douglas N, Helma C, Rautenberg M, Jeliazkova N, Jeliazkov V, Nikolova I, Benigni R, Tcheremenskaia O, Kramer S, Girschick T, Buchwald F, Wicker J, Karwath A, Gütlein M, Maunz A, Sarimveis H, Melagraki G, Afantitis A, Sopasakis P, Gallagher D, Poroikov V, Filimonov D, Zakharov A, Lagunin A, Gloriozova T, Novikov S, Skvortsova N, Druzhilovsky D, Chawla S, Ghosh I, Ray S, Patel H, Escher S (2010) Collaborative development of predictive toxicology applications. J Cheminform 2:7
143. Ekins S, Williams AJ (2010) Precompetitive preclinical ADME/Tox data: Set it free on the web to facilitate computational model building and assist drug development. Lab Chip 10:13–22
144. Gupta RR, Gifford EM, Liston T, Waller CL, Hohman M, Bunin BA, Ekins S (2010) Using open source computational tools for predicting human metabolic stability and additional absorption, distribution, metabolism, excretion, and toxicity properties. Drug Metab Dispos 38:2083–2090

Chirality and Its Role in Environmental Toxicology

Imran Ali, Hassan Y. Aboul-Enein, Mohd Marsin Sanagi, and Wan Aini Wan Ibrahim

Abstract The role of chirality is crucial in our lives as it affects various processes and phenomenon in the earth's ecosystem. The chiral pollutants are widely distributed in water, soil, sand, air, and biota. Most notorious chiral pollutants are pesticides, biphenyls, polychlorinated hydrocarbons, and some drug residues. Enantiomers of chiral xenobiotics have different toxicities, and, hence, determination of their enantioselective toxicities is essential by the environmental point of views. The toxicities of enantiomers of some chiral pollutants have been established. This chapter describes the origin, chemistry, and environmental aspects of chirality. Attempts have been made to discuss the distribution and toxicities of various chiral pollutants in the environment.

Keywords Chirality · Chiral pollutants · Distribution of the chiral pollutants · Enantioselective toxicities

I. Ali
Department of Chemistry, Jamia Millia Islamia (Central University), New Delhi, India

H.Y. Aboul-Enein (✉)
Pharmaceutical and Medicinal Chemistry Department, Pharmaceutical and Drug Industries Research Division, National Research Centre, Dokki, Cairo 12311, Egypt
e-mail: haboulenein@yahoo.com

M.M. Sanagi and W.A.W. Ibrahim
Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 81310, UTM Johor Bahru, Johor, Malaysia

A. Luch (ed.), *Molecular, Clinical and Environmental Toxicology*,
Experientia Supplementum 101, DOI 10.1007/978-3-7643-8340-4_14,

Introduction

For few decades, the environmental pollution has become the issue of focus among scientists, academicians, environmentalists, and regulatory authorities. There are many kinds of pollutants; some organic contaminants are most dangerous due to their chronic toxicities and carcinogenic nature. The most commonly found organic pollutants are pesticides, phenols, plasticizers, polychlorinated, and polycyclic aromatic hydrocarbons (PAHs) [1–4]. The presence of such pollutants in our earth ecosystem is dangerous and many methods have been reported for their monitoring. It is very important to highlight that the reported methods provide the total concentrations of pollutants but they do not distinguish which mirror images of pollutants are present and which are harmful in the case of chiral contaminants. Therefore, during the last few years, scientists and regulatory authorities are in the demand of the data on concentrations and toxicities of the chiral pollutant mirror images. This is an essential, urgent, and demanding field in the present century. For the preparation of this chapter, we have searched the literature thoroughly and observed that only few groups are working in this area. This chapter describes the role of chirality in the environment, especially the distribution and toxicities of chiral pollutants.

Origin of the Chirality

Before discussing the distribution and toxicities of chiral pollutants, it is essential to discuss some aspects of chirality so that readers can realize the importance of chirality in the environment. Basically, chirality has been derived from the Greek word *kheir* for handedness and, first of all, used by Lord Kelvin in 1883 [5]. Any molecule deprived of plane, center, and axis of symmetry exists in more than one form; these are called chiral objects or enantiomers; enantiomers that are nonsuperimposable mirror images of each other are called as chiral objects. The role of chirality is very important in our lives and still has not been fully explored. Chirality is found in a wide range of objects starting from elementary constituents of our body structure [6]. There are several examples of the chirality in our environment, i.e., burial chamber mural paintings in Egypt [5], 540 galaxies listed in *Carnegie Atlas of Galaxies* [7], and helical structures of plants and animals. Briefly, the chirality exists almost everywhere in this universe and is associated with the origin of the earth and life [8].

Chemistry of the Chirality

In 1809, Haüy [9] evolved chemical utility of the chirality, who postulated that, from crystal cleavage observations, a crystal and each constituent space-filling molecules are images of each other in overall shape. In 1848, Pasteur described the different destruction rates of *dextro* and *levo* ammonium tartrate by the mould *Penicillium glaucum* [10].

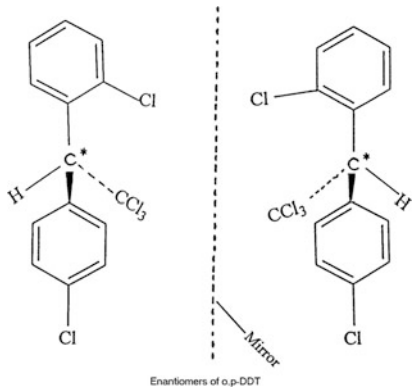


Fig. 1 Enantiomers of *o*, *p*′-DDT (1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*p*′-chlorophenyl)ethane)

The tetrahedral arrangement of carbon with different four groups makes the whole pollutant asymmetric in structure, and such pollutant differs in three dimensional configurations and exists in two forms, which are mirror images of each other (Fig. 1). These mirror images are called optical isomers, stereoisomers, enantiomers, enantiomorphs, antipodes, or chiral molecules. The phenomenon of the existence of the enantiomers is called as stereoisomerism or chirality. The 50:50 ratios of the enantiomers are called racemic mixture, which do not rotate the plane-polarized light. The number of the enantiomers may be calculated by 2^n, where n is the number of the chiral centers within the respective molecule. In the beginning, the optical isomers were distinguished with (+) and (−) signs or *d* (*dextro*) and *l* (*levo*), indicating the direction in which the enantiomers rotate the plane-polarized light. (+) or *d* stands for a rotation to the right (clockwise), whereas (−) or *l* indicates a rotation to the left (anticlockwise). The main drawback of such an assignment is that one cannot derive the number of chiral centers from it. This is possible when applying the well-known *R*/*S* notation given by Cahn and Ingold, which describes the absolute configuration (the spatial arrangement of the substituents) around the asymmetric carbon atom of the pollutant (molecule).

Environmental Aspects of the Chirality

The most notorious environmental pollutants are pesticides, which are about 25% chiral molecules [11]. Polyaromatic hydrocarbons may also be chiral pollutants. It has been observed that one of the enantiomers of the chiral pollutant may be more toxic, and, hence, both enantiomers may have different toxicities [5, 12]. This is an important information to scientists when performing environmental analyses. Biological transformation of the chiral pollutants can be stereoselective; uptake, metabolism, and excretion of enantiomers may be very different [12, 13]. Therefore, the enantiomeric composition of the chiral pollutants may be changed through these processes. Metabolites of the chiral compounds are often chiral as well. Moreover, some of the achiral

pollutants degrade into the chiral metabolites. For example, γ-hexachlorocyclohexane (γ-HCH) and atrazine degrade into γ-pentachlorocyclohexene (γ-PCCH) and 2-chloro-4-ethylamino-6-(1-hydroxy-2-methylethyl-2-amino)-1,3,5-triazine racemic mixtures, respectively. It has also been reported that the enantiomers may react at different rates with achiral molecules in the presence of chiral catalysts [4]. Since constituents of living organisms are usually chiral, there are greater chances of the chiral pollutants to react at different rates. To predict the exact chiral pollution load determination of enantioselective toxicities and concentrations of the enantiomers is thus required and an essential need.

Distribution of the Chiral Pollutants in the Environment

Both point and nonpoint sources are major sources for pollution in the environment. The most commonly found chiral pollutants are given in Table 1. These compounds are widely distributed in our ecosystem. Marine water has been reported as polluted due to heptachlor *exo*-epoxide (a metabolite of heptachlor); α-, β-, and γ-HCH; toxaphene; and phenoxyalkanoic acid herbicides. There are only few papers published [5] on the ground water contamination by pesticides and other toxic organic pollutants [3]. Weigel [14] reported on the presence of several drugs in waste water. Buser *et al.* [15] identified ibuprofen, a nonsteroidal anti-inflammatory drug, in waste and river waters. Recently, Ali *et al.* [16] reviewed the literature on the distribution of drugs in the environment.

Vetter *et al.* [17] determined toxaphene in Canadian lake sediments and chloroborane congeners in the sediment from the toxaphene-treated Yukon lake [18]. Rappe *et al.* [19] reported the presence of chiral pesticides in the sediment of the Baltic Sea, and Benicka *et al.* [20] identified PCBs in sediments of a river. Wong *et al.* [21] looked into the enantiomeric ratios of eight PCB species in the sediments from selected sites in the USA. Biselli *et al.* [22] carried out a comprehensive study on the distribution of the chiral musks in sediments of various waste water plants. Moisey *et al.* [23] determined the concentrations of α-, β-, and γ-HCH isomers and enantiomers in sediments obtained from the North Sea. Aigner *et al.* [24] described the enantiomeric ratio of the pesticide chlordane in the soil of Midwestern USA. The pesticides detected in these samples were chlordane, heptachlor, and heptachlor *exo*-epoxide. Wiberg *et al.* [25] described organochlorine pesticides in 32 agricultural and 3 cemetery soils from Alabama. Lewis *et al.* [11] detected the dichlorprop pesticide in Brazilian soils, and White *et al.* [26] identified *cis*- and *trans*-chlordanes in the soil of a green house unit.

Besides water, soil, and sediments, chiral pollutants have also been detected in the atmosphere. The concentrations of chiral pollutants being found varied from place to place. Ridal *et al.* [27] detected α-HCH in air above the water surface of Ontario Lake. Ulrich and Hites [28] reported the presence of chlordane in air samples near the Great Lakes. Aigner *et al.* [24] reported enantiomeric ratios of the chlordane pesticide in the air of Midwestern USA. Similarly, Bidleman *et al.* [29] collected air samples from Corn Belt, South Carolina, and Alabama areas.

Table 1 List of some common chiral pollutants [4]

AHTN (tonalide: 1-(5,6,7,8-tetrahydro-3,5,5,6,8,8-hexamethyl-2-naphthalenyl)ethanone)
ATTI (traseolide: 1-(2,3-dihydro-1,1,2,6-tetramethyl-3-isopropyl-1*H*-inden-5-yl)ethan-1-one)
AHDI (phantolide: 1-(2,3-dihydro-1,1,2,3,3,6-hexamethyl-1*H*-inden-5-yl)ethanone)
Anatoxin-a
Acephate
Biollethrin
Bromacil
Bromocyclane (bromodan)
Bromocyclen
Chlordane (*cis*, *trans,* and other congeners)
Chlordane and metabolites
Clofibric acid
Cruformate
Crotoxyphos
Crufomate
Dialifor
o,*p*′-DDT (1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*p*′-chlorophenyl)ethane)
o,*p*′-DDD (1,1-dichloro-2-(*o*-chlorophenyl)-2-(*p*′-chlorophenyl)ethane)
α-, β-, γ-, δ-HCH (α-, β-, γ-, and δ-hexachlorocyclohexane)
DCPP (dichlorprop: 2-(2,4-dichlorophenoxy)propionic acid)
Deltamethrin
Endosulfan
Fonofos
Fenamiphos
Fensulfothion
HHCB (galaxolide: 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[*g*]-2-benzopyran)
Heptachlor
Heptachlor *exo*-epoxide
Homoanatoxin-a
Isophenphos
Isomalathion
Ibuprofen and metabolites
MCCP (2-(4-chloro-2-methylphenoxy)propionic acid)
MDCPP (methyl dichlorprop: methyl 2-(2,4-dichlorophenoxy)propionic acid)
Methamidophos
Malaxon
Malathion
Methamidophos
Naloxone
Oxychlordane
β-, γ-PCCH (β-, γ-pentachlorocyclohexene)
Photoheptachlorepoxide
Photochlordane
Profenofos
Prothiophos
PCBs (polychlorinated biphenyls)
PCB methyl sulfones ($MeSO_2$-PCBs)
Ruelene (4-*tert*-butyl-2-chlorophenylmethyl-*N*-methylphosphoramidate)
Saxitoxin
Trichloronate
4-Tolylethyl sulfoxide
Tetrodotoxin
Toxaphene
Thalidomide
Trichlorfon

Table 2 Chiral pollutants in various water resources [4]

Chiral pollutant	Water resource
Acetachlor	River water
Chlordane	River water
Clofibric acid	River water
DCPP	Sea water
Endosulfan	River water
α-HCH	Sea water
	Rain water
Heptachlor	River water
Ibuprofen	River water
Metolachlor	River water
β-PCCH	Rain water
γ-PCCH	Sea water
Polyaromatic musks	River water

The authors reported the presence of *cis*-chlordane, *trans*-chlordane, heptachlor, and heptachlor *exo*-epoxide in these samples. Other authors who described the presence of chiral pesticides in air samples are Wiberg *et al.* [30] (chlordane) and Buser and Müller [31] (heptachlor and chlordane).

Moreover, chiral xenobiotics have been routinely identified in earth's biota. Different chiral ratios of different pollutants were detected in various organs of seals, Eider ducks, polar bears, whales, pelagic zooplankton, arctic cod, sea birds, fishes, bivalves, crayfish, water snakes, barn swallows, sheep, roe deer, and even humans [32–40]. For a ready reference, Tables 2 and 3 describe the distribution of chiral pollutants in water and biota of our ecosystem. The different chiral ratios of toxaphene, *cis*- and *trans*-chlordane, and heptachlor *exo*-epoxide in air, sediment, soil, and plants are given in Tables 4–7.

Toxicities of Chiral Pollutants

Only little information is available on the enantioselective toxicity of pollutants. Basically, differences in the bioaffinity of the enantiomers to a binding site on an enzyme or receptor surface are responsible for different toxicities. Such differences may reveal in terms of distribution rates, compound's metabolism, and excretion; in antagonistic actions relative to each other; or in their individually different tissue-specific toxicological properties. The enantioselective toxicities of chiral pollutants are discussed in the following sections.

Enantioselective Toxicities of Pesticides

Möller *et al.* [41] described the different carcinogenic potencies and growth stimulation of α-HCH enantiomers in primary rat hepatocytes by reporting 100% cell death in the presence of 3.0×10^{-4} M (+)-α-HCH. Contrarily, (−)-α-HCH only induced 75% toxicity at the same concentration. By using concentrations of

Table 3 Chiral pollutants in different biota [4]

Chiral pollutant	Ecosystem component
AHTN	Rudd
	Trench liver
	Crucian carp liver
	Eel
	Mussels
trans-Chlordane	Baltic herring
	Baltic salmon
	Baltic seal
cis-Chlordane	Baltic herring
	Baltic salmon
α-HCH	Mussel
	Eider duck (liver)
	Eider duck (kidney)
	Eider duck (muscle)
	Seal liver
	Seal (blubber)
	Seal (brain)
	Seal (lung)
	Female fur seal (milk)
	Whale blubber
	Flounder (liver)
	Cod liver oil
HHCB	Rudd
	Trench liver
	Crucian carp liver
	Eel
	Mussels
Heptachlor *exo*-epoxide	Sea gulls egg
Octachlordane MC4	Baltic herring
	Baltic salmon
	Baltic seal
	Antarctic penguin
Octachlordane MC5	Baltic herring
	Baltic salmon
	Baltic seal
	Antarctic penguin
Octachlordane MC7	Baltic herring
	Baltic salmon
	Baltic seal
	Antarctic penguin
Oxychlordane	Sea gulls egg
β-PCCH	Flounder

5.0×10^{-5} M of both enantiomers, significant increases in mitosis occurred in the presence of the (+)-α-HCH enantiomer (factor 2.4) as compared with a 1.7-fold stimulation by (−)-α-HCH enantiomer. Concentration-dependent cell death rates observed in primary cultures of rat hepatocytes treated with (+)- or (−)-α-HCH and the corresponding stimulation of mitosis in these cells are depicted in Fig. 2.

Table 4 Chiral ratio of toxaphene congeners b7-1001 and b7-923 in sediments from the Canadian Hanson Lake [4]

Year of sampling	B7-1001	B6-923
1935	<1.0	0.97
1946	0.80	1.00
1954	0.81	1.01
1959	0.82	0.97
1964	0.82	1.06
1968	0.81	0.98
1973	0.78	0.98
1979	0.77	0.98
1984/7	–	0.99
1992	0.71	0.96

Table 5 Chiral ratio of *cis*- and *trans*-chlordane pesticides in soil samples [4]

Soil samples	Enantiomeric ratio of *cis*-chlordane	Enantiomeric ratio of *trans*-chlordane
Prebulk (prior to plantation)	1.22	0.861
Postbulk (after to plantation)	1.25	0.872
Near plant root	1.24	0.852

Table 6 Chiral ratio of chlordane, heptachlor, and heptachlor *exo*-epoxide in air from the Corn Belt region, South Carolina, and Alabama [4]

Location	*trans*-Chlordane	*cis*-Chlordane	Heptachlor chlordane	Heptachlor *exo*-epoxide
Alabama				
Ambient	0.98	1.01	–	–
Indoor	0.98	1.00	–	–
Corn Belt				
Above	0.74	1.11	–	–
Ambient	0.93	1.04	–	–
Indoor	0.99	0.98	–	–

Miyazaki *et al.* [42] studied the enantioselective toxicities of cyclodiene pesticides (e.g., chlordiene, chlordiene epoxide, and heptachlor *exo*-epoxide) on male German cockroach insects (*Blattella germanica*). The authors reported that (+)-chlordiene, (−)-chlordiene epoxide, and (+)-heptachlor *exo*-epoxide enantiomers exhibited stronger toxicity when compared to their corresponding antipodes (Table 8). The toxicity was expressed in percent of dead animals 24 h after the application of the compound. From the results obtained, it can be concluded that (+)-chlordiene, (−)-chlordiene epoxide, and (+)-heptachlor *exo*-epoxide are more toxic in these insects than their enantiomers. The $LD_{28.6}$ value of (+)-chlordiene was 129, while LD_{50} values of (−)-chlordiene epoxide and its racemate were 76 and 157, respectively, indicating the differences of enantioselective toxicities of these insecticides.

Furthermore, Miyazaki *et al.* [43] reported the different enantioselective toxicities of heptachlor and 2-chloroheptachlor pesticides on the same cockroach species. LD_{50} values for these pesticides were calculated after 24 h (Table 9). It has been reported that only heptachlor and 2-chloroheptachlor showed toxicities, while 3-chloroheptachlor was nontoxic. LD_{50} values for (−)-, (+)-, and (±)-heptachlor were 5.32, 3.38, and 2.64,

Table 7 Chiral ratio of *cis*- and *trans*-chlordane in different plants [4]

Plants	Enantiomeric ratio of *cis*-chlordane	Enantiomeric ratio of *trans*-chlordane
Cucumber		
Roots	0.54	0.42
Stem	0.52	0.38
Leaves	0.52	0.37
Whole fruit	0.51	0.25
Fruits peel	0.48	0.30
Fruit flesh	0.50	0.22
Lettuce		
Roots	0.55	0.46
Leaves	0.54	0.41
Pumpkin		
Roots	0.51	0.51
Stem	0.46	0.49
Leaves	0.54	0.48
Whole fruit	0.56	0.47
Fruits peel	0.55	0.47
Fruit flesh	0.56	0.47
Pepper		
Roots	0.55	0.48
Stem	0.56	0.54
Leaves	0.52	0.51
Spinach		
Roots	0.53	0.46
Leaves	0.58	0.45
Tomato		
Roots	0.55	0.37
Stem	0.59	0.30
Leaves	0.52	0.30
Zucchini		
Roots	0.55	0.42
Stem	0.58	0.36
Leaves	0.58	0.47
Whole fruit	0.60	0.46
Fruits peel	0.59	0.40
Fruit flesh	0.62	0.48

respectively. On the other hand, LD_{50} values calculated for (−)-, (+)-, and (±)-2-chloroheptachlor were 100, 50, and 20, respectively. Therefore, it may be concluded that the toxicities of the (+)-enantiomers of heptachlor and 2-chloroheptachlor are greater than that of their corresponding (−)-enantiomers. Based on these results, the theoretical LD_{50} values of the racemic mixtures of heptachlor and 2-chloroheptachlor should be 4.35 and 75.0, respectively. However, the observed LD_{50} values are lower than the theoretical values (Table 9). Therefore, it may be concluded that the toxic potency of one enantiomer is being increased due to the presence of the other.

McBlain and Lewin [44] reported (−)-*o*,*p*′-DDT as a more active estrogen-mimic species in rats than the (+)-enantiomer. Hoekstra *et al.* [45] described a yeast-based assay to assess the enantiomer-specific transcriptional activity of *o*,*p*′-DDT via interaction with the human estrogen receptor (hER). While the (−)-enantiomer strongly

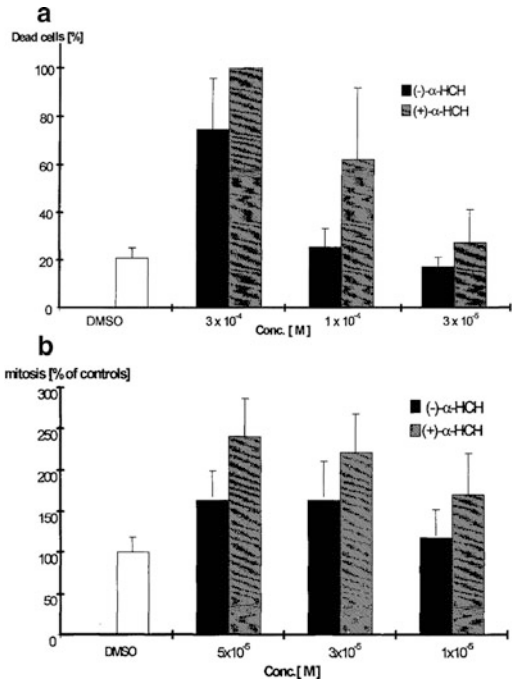


Fig. 2 Differences in the biological effects of the (+)- and (−)-enantiomers of α-HCH (α-hexachlorocyclohexane). (**a**) Toxicity in primary cultures of rat hepatocytes and (**b**) effects on the stimulation of mitosis in these cells (from [41] with reprint permission of Eco-Informa Press)

Table 8 Chiral toxicities of chlordiene, chlordiene epoxide, and heptachlor *exo*-epoxide insecticide enantiomers in the German cockroach *Blattella germanica* (% of dead animals after 24 h) (according to [42])

Insecticide	Dose (μg/g)					LD (μg/g)
	300	233	178	126	94.4	$LD_{28.6}$
(+)-Chlordiene	100	94.4	72.2	33.3	11.1	129
(±)-Chlordiene	28.6	–	–	–	–	–
(−)-Chlordiene	0	–	–	–	–	–

Insecticide	Dose (μg/g)				LD (μg/g)
	200	133.3	88.9	59.3	LD_{50}
(−)-Chlordiene epoxide	86.7	73.3	66.7	33.3	76
(±)-Chlordiene epoxide	66.7	40.0	13.3	0.0	157
(+)-Chlordiene epoxide	0	–	–	–	–

Insecticide	Dose (μg/g)					LD (μg/g)
	6.0	3.0	1.5	0.75	0.375	LD_{50}
(+)-Heptachlor *exo*-epoxide	100	86.7	46.7	33.3	6.7	1.29
(±)-Heptachlor *exo*-epoxide	93.3	80.0	33.3	13.3	0	1.82
(−)-Heptachlor *exo*-epoxide	93.3	46.7	13.3	0	0	2.98

Table 9 Chiral toxicities of heptachlor and 2-chloroheptachlor enantiomers in the German cockroach *Blattella germanica* (% of dead animals after 24 h) (according to [43])

Insecticide	Dose (μg/g)						LD_{50} (μg/g)
	18.0	10.8	6.48	3.88	2.32	1.39	
(+)-Heptachlor	93.3	66.7	43.3	0	0	0	3.38
(±)-Heptachlor	86.7	93.3	60.0	25.0	0	0	2.64
(−)-Heptachlor	90.0	46.7	36.7	0	0	0	5.32
Insecticide	Dose (μg/g)						LD_{50} (μg/g)
	200	100	50	25	12.5		
(+)-2-Chloroheptachlor	100	100	100	60	40		20
(±)-2-Chloroheptachlor	100	80	50	10	10		50
(−)-2-Chloroheptachlor	40	40	0	0	0		100

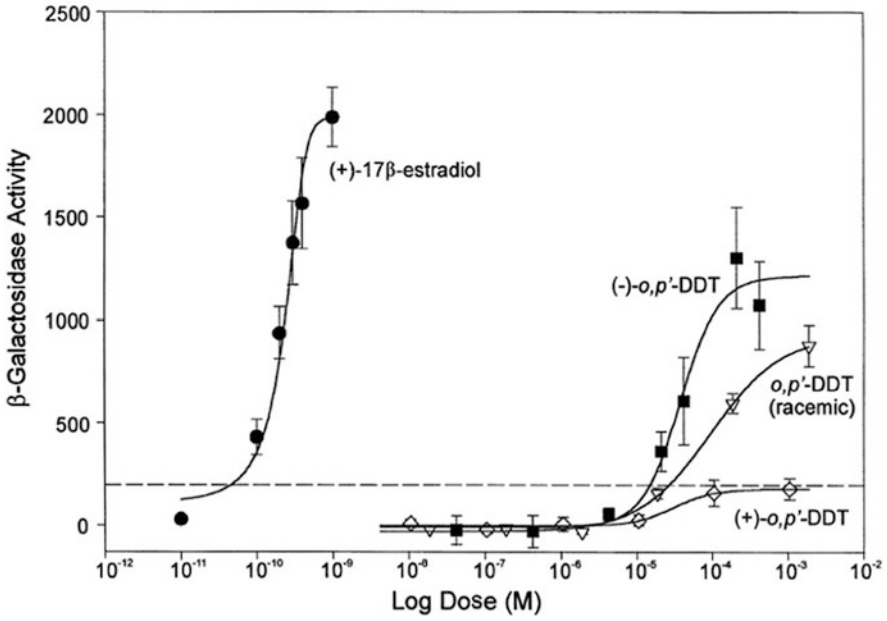


Fig. 3 Differences in enantiomer-specific transcriptional activity of *o,p′*-DDT via interaction with the human estrogen receptor (hER). Binding affinities of the (+)- and (−)-enantiomers and the racemic mixture were measured via β-galactosidase activity (from [45] with reprint permission of Elsevier)

induced measureable hER activity, the corresponding potency of the (+)-*o,p′*-DDT was negligible. However, high concentrations of the (+)-enantiomer influenced (decreased) the transcriptional activity of the (−)-*o,p′*-DDT. The dose-dependent reporter gene (β-galactosidase) activity is shown in Fig. 3.

Miyazaki *et al*. [46, 47] reported on the enantioselective differences in the toxicities of methamidophos (*O,S*-dimethyl phosphoramidothiodate) and acetaphate (*O,S*-dimethyl-*N*-acetylphosphoramidothiodate) to houseflies. In houseflies, the (+)-enantiomers are more potent than their (−)-counterparts. By contrast, the (−)-enantiomers

were found more toxic to German cockroaches (*B. germanica*), albeit LD_{50} values were close for both enantiomers. In addition, the (−)-enantiomer resulting from sulfoxidation of propaphos was found more potently inhibiting cockroaches and—at the biochemical level—the bovine erythrocyte acetylcholinesterase (AChE) when compared with its (+)-enantiomer [47]. Furthermore, the authors studied the toxicities of these two enantiomers on houseflies and green leaf hoppers and reported only little differences in the toxicities in these insects. Phosphor-containing pesticides were introduced in the 1950s for insect control in fruits, vegetables, and other crops.

Lang *et al.* [48] described the conversion of atrazine into the racemic mixture of 2-chloro-4-ethylamino-6-(1-hydroxy-2-methylethyl-2-amino)-1,3,5-triazine by liver microsomes of rats, pigs, and humans. The authors reported on the dominance of the *R*-enantiomer in humans, while the higher concentrations of the *S*-enantiomer were observed in rats and pigs. A species-dependent enantioselective formation of this metabolite with *S*/*R* ratios of 76:24 in rats, 49:51 in pigs, and 28:72 in humans was stated. Similarly, *trans*-nonachlor, a major constituent of technical chlordane, is achiral, and the replacement of chlorine substituents by another atom or group can produce a chiral derivative. Further, malathion usually is biotransformed into racemic malaxon that exhibits anti-AChE (insecticidal) activity. For bovine erythrocyte cholinesterase, the antagonistic activity of the *R*-enantiomer is 22 times greater than for the *S*-enantiomer [49, 50].

Enantioselective Toxicities of Polychlorinated Biphenyls

Polychlorinated biphenyls (PCBs) are the most notorious class of chlorinated chiral pollutants. Although their use has been banned in many countries since the 1970s, these compounds still represent an important class of priority pollutants due to their long persistence, bioaccumulation, and toxicity [51]. About 209 PCB congeners are known, and out of them 78 are chiral in nature. Again out of these 78 PCB congeners only 19 form stable enantiomers (atropisomers) [52]. Different toxicities of these chiral PCBs have been described in terms of porphyria, teratogenicity, endocrine and reproductive malfunctions, *etc*. It has been reported that non-*ortho* coplanar PCBs exhibit the highest toxicities followed by the moderately toxic mono-*ortho* coplanar congeners, while di-*ortho*-substituted PCBs turned out to be less toxic. Ahlborg *et al.* [53] presented a toxic equivalency model to describe the toxicities of PCB congeners. The authors calculated the toxic equivalency factors (TEFs) for individual PCBs. Each PCB has been assigned a TEF value based on its toxicity relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which has by definition a TEF of 1.00 (100%). Püttman *et al.* [54] reported PCB139 and PCB197 congeners as effective inducers of drug-metabolizing enzymes (e.g., cytochrome P-450 monooxygenases, CYPs; *N*-demethylase; and aldrin epoxidase). The authors described the (+)-enantiomer of PCB139 as the stronger inducer in comparison to the (−)-enantiomer. Contrarily, the racemic mixtures of PCB197 and its individual enantiomers are only weak inducers of these enzymes. Furthermore, in

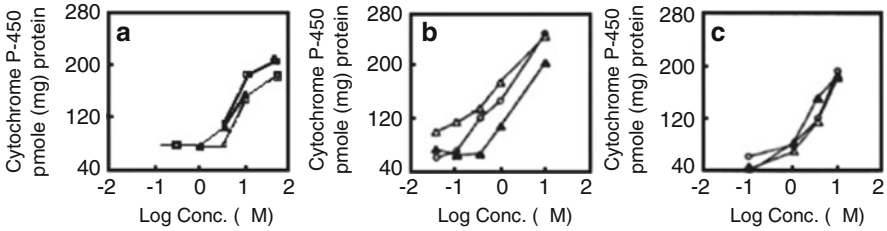


Fig. 4 Effects of the enantiomers of (**a**) PCB88, (**b**) PCB139, and (**c**) PCB197 congeners on the induction of total cytochrome P-450 enzymes (CYPs); (*triangle*) (+)-enantiomer, (*filled triangle*) (−)-enantiomer, and (*circle*) racemic mixture (from [55] with reprint permission of Elsevier)

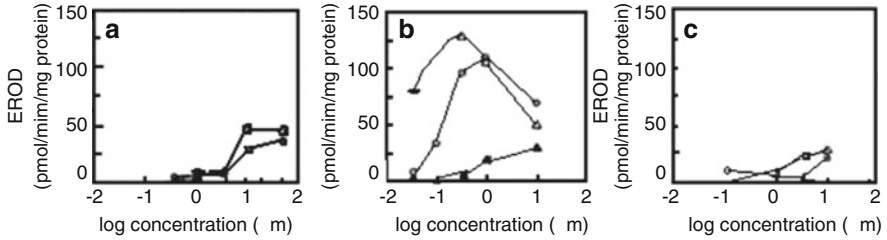


Fig. 5 Effects of the enantiomers of (**a**) PCB88, (**b**) PCB139, and (**c**) PCB197 congeners on the induction of ethoxyresorufin-*O*-deethylase (EROD) activity; (*triangle*) (+)-enantiomer, (*filled triangle*) (−)-enantiomer, and (*circle*) racemic mixture (from [55] with reprint permission of Elsevier)

1991, the same group reported activities related to the induction of CYPs, that is, the activity of ethoxyresorufin-*O*-deethylase (EROD) and benzphetamine-*N*-demethylase (BPDM) [55]. They demonstrated that EROD activity is induced to much greater extent by (+)-enantiomers of all of the congeners studied with no activities of the (−)-enantiomers of PCB88 and PCB197. The effects of the enantiomers of PCB88, PCB139, and PCB197 on the induction of total CYP enzymes and EROD and BPDM activities are shown in Figs. 4–6, respectively.

The effects of the enantiomers of PCB88, PCB139, and PCB197 on the accumulation of protoporphyrin and uroporphyrin (URO) chick embryo liver cell cultures are summarized in Table 10. The results indicate that URO accumulation occurred only at high concentrations (i.e., $\geq$1.0 μM) of PCB88 and PCB197, but at low concentrations of the PCB139 congener [$\geq$0.034 μM for (+)-PCB139 and $\geq$0.34 μM for (−)-PCB139]. The strongest URO accumulation occurred with PCB139, with 64% URO generated by the (+)-enantiomer and 47% URO generated by the (−)-enantiomer at the highest concentration tested.

PCB methyl sulfones ($MeSO_2$-PCBs) are metabolites of PCBs generated via the mercapturic acid pathway. Cleavage of the sulfur–carbon bond in the cysteine moiety, methylation, and oxidation of the methyl sulfide has been described by Bakke and Gustafsson [56]. These metabolites are more persistent and less hydrophobic

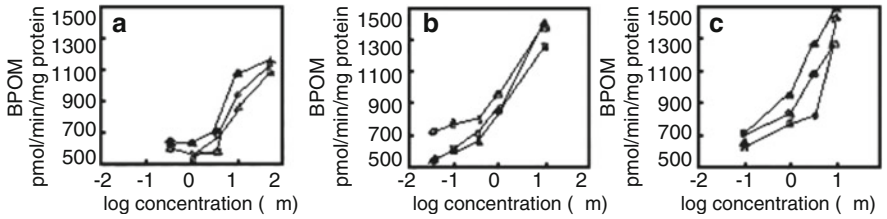


Fig. 6 Effects of the enantiomers of (**a**) PCB88, (**b**) PCB139, and (**c**) PCB197 congeners on the induction of benzphetamine-*N*-demethylase (BPDM) activity; (*triangle*) (+)-enantiomer, (*filled triangle*) (−)-enantiomer, and (*circle*) racemic mixture (from [55] with reprint permission of Elsevier)

Table 10 Concentrations of enantiomers of PCB88, PCB139, and PCB197 and induced accumulation of protoporphyrin and uroporphyrin in chick embryo liver cell culture [55]

Concentration (μM)	% Protoporphyrin		% Uroporphyrin (URO)	
	(+)-PCB88	**(−)-PCB88**	**(+)-PCB88**	**(−)-PCB88**
0.34	90.0	88.3	3.9	4.2
1.0	85.4	87.1	8.2	5.2
3.4	65.0	83.1	17.1	7.0
10.0	51.0	63.2	24.5	20.7
50.0	23.7	20.5	34.1	45.1
	(+)-PCB139	**(−)-PCB139**	**(+)-PCB139**	**(−)-PCB139**
0.034	85.7	89.6	7.0	4.1
0.1	83.5	89.1	8.6	4.1
0.34	84.8	84.6	8.5	8.0
1.0	68.3	81.5	22.1	10.7
10.0	19.8	37.9	63.5	46.6
	(+)-PCB197	**(−)-PCB197**	**(+)-PCB197**	**(−)-PCB197**
0.1	90.8	91.8	2.9	2.6
1.0	88.8	91.4	4.8	2.3
3.4	83.9	85.9	7.5	6.3
10.0	70.8	61.0	17.6	26.2

than their corresponding parents, which make them long-lasting contaminants of the biosphere. Several $MeSO_2$-PCBs have been shown to strongly induce CYP activity such as CYP2B2, 3A2, and 2C6. A study on the influence of $MeSO_2$-PCBs in the reproduction of minks (*Mustela vison*) indicated that $MeSO_2$-PCB and 1,1-dichloro-2-(*o*-chlorophenyl)-2-(*p*′-chlorophenyl)ethylene (DDE) methyl sulfone mixtures increased the litter size in these animals [57]. A strong respiratory distress and alterations in the immune status of yusho patients in Japan have been related to $MeSO_2$-PCBs [58]. There are several reports describing the toxicities of $MeSO_2$-PCBs, but, unfortunately, no report is available on the enantioselective toxicities of $MeSO_2$-PCBs. Furthermore, a Swedish and German collaborative project started in 1997 with the aim of studying enantioselective accumulation of $MeSO_2$-PCBs in the liver of humans and rats [36, 59], but data on the enantioselective toxicities are still missing.

Enantioselective Toxicities of Polycyclic Aromatic Hydrocarbons

Among the most toxic PAHs in the environment are β-naphthoflavone, benzo[*a*]-pyrene (BP), and anthracene and their derivatives. Toxicities of the racemic metabolites of PAHs are known for a long time, but only few reports are available on their enantioselective cytotoxic, mutagenic, and carcinogenic effects. In 1977, Levin *et al.* [60] studied the carcinogenic activity of *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]-pyrene (BP-7,8-dihydrodiol) on CD-1 mouse skin with 50, 100, and 200 nmols of each of the (+)- and (−)-enantiomers. It was observed that the (−)-enantiomer was more toxic than the (+)-enantiomer at all concentrations. The maximum tumor formation was observed after 21 weeks, with a 5–10 times higher carcinogenic activity of the (−)-enantiomer in comparison to the (+)-enantiomer. Furthermore, the same authors described the effect of BP-7,8-dihydrodiol on the skin of newborn mice, concluding the greater toxicity of the (−)-enantiomer. While the parent BP is not a carcinogen by itself, its metabolic products such as diol-epoxides are highly potent [61–66]. Two possible diastereoisomers originating from *trans*-BP-7,8-dihydrodiol were characterized [67], and one of them was shown to be an ultimate carcinogen in newborn mice [68, 69]. The diastereomer BP-7β,8α-dihydrodiol-9α,10α-epoxide (*anti*-BPDE) has been characterized as potent mutagen in bacteria and certain mammalian cells [70–72]. Wood *et al.* [73, 74] reported the (+)-enantiomer [i.e., (+)-BP-7β,8α- diol-9α,10α-epoxide, (+)-*anti*-BPDE] being four times more toxic in Chinese hamster cells than its (−)-antipode [(−)-*anti*-BPDE]. Slaga *et al.* [75] also studied the enantioselective carcinogenesis of *anti*-BPDE on mouse skin. According to the authors, the carcinogenic potency of the (+)- and (−)-*anti*-BPDE enantiomers were 60% and 2%, respectively. The results of this study are depicted in Fig. 7.

The stereoselective metabolism of BP toward its ultimate carcinogen occurs as follows: BP→BP-7,8-oxide→*trans*-BP-7,8-dihydrodiol→BP-7,8-diol-9,10-epoxide (BPDE). Chang *et al.* [76] reported that the (−)-enantiomer of BP-4,5-oxide was 1.5–5.5-fold more mutagenic than the (+)-enantiomer in bacterial strains of *Salmonella typhimurium* (TA98, TA100, TA1537, TA1538) and in Chinese hamster V79 cells. The authors reported when mixtures of the enantiomers were studied in V79 cells, synergistic cytotoxic and mutagenic responses could be observed. The most cytotoxic and mutagenic effects occurred with a 3:1 mixture of the (−)- and (+)-enantiomers of BP-4,5-oxide. Levin *et al.* [77] described that the (+)-BP-7,8-oxide showed greater enantioselective toxicity in the skin of newborn mice (see Table 11). It is interesting to note that the tumor formation potencies of BP-7,8-oxide were in the order: racemic mixture > (+)-enantiomer > (−)-enantiomer. The higher toxicity of the racemic mixture might be the result of catalytic interferences between the enantiomers.

Wood *et al.* [78] studied the enantioselective toxicities of four isomers of chrysene-1,2-diol-3,4-epoxide in bacterial (histidine dependent) strains of *S. typhimurium* and in mammalian (Chinese hamster V79) cells. In strain TA98 of *S. typhimurium*, the (−)-*anti*-chyrsene-1,2-diol-3,4-epoxide was 5–10 times more toxic compared to the other three isomers. However, in strain TA100 of these bacteria and in Chinese hamster V79 cells, (+)-*anti*-chyrsene-1,2-diol-3,4-

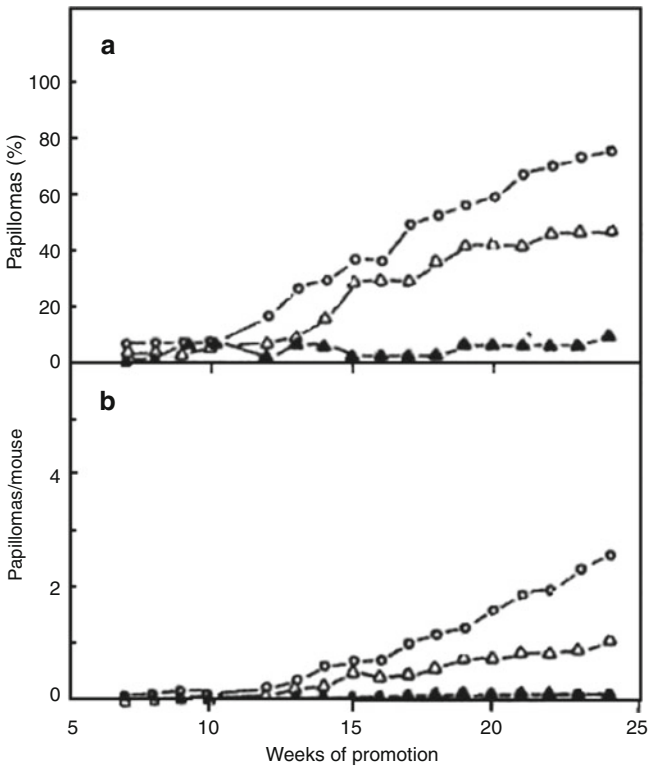


Fig. 7 Skin tumor-initiating activities of (*circle*) (+)-BP-7β,8α-diol-9α,10α-epoxide [(+)-*anti*-BPDE], (*filled triangle*) (−)-BP-7α,8β-diol-9β,10β-epoxide [(−)-*anti*-BPDE], and (*triangle*) racemic BP-7,8-diol-9,10-epoxide [(±)-*anti*-BPDE] in female CD-1 mice. A dose of 200 nmol of each compound was applied. (**a**) Percentage of papillomas; (**b**) Papillomas per mouse (from [75] with permission of American Association for Cancer Research—AACR)

Table 11 Chiral tumorigenicity of BP-7,8-oxide enantiomers (according to [77])

BP-7,8-oxide	Dose (nmol)	% of mice with tumors	No. of tumors per mouse
In adult mice			
(−)	100	11	0.11
	400	36	0.43
(+)	100	18	0.54
	400	55	1.03
(±)	100	50	0.83
	400	60	1.67
In newborn mice treated with 100, 200, and 400 nmol at the 1st, 8th, and 15th day of life			
(−)		21[a]	0.33
		20[b]	0.20
(+)		86[a]	1.82
		83[b]	2.57
(±)		93[a]	3.89
		86[b]	3.39

Female[a] and male[b] mice, respectively

Table 12 Chiral tumorigenicity of *trans*-1,2-dihydroxy-1,2-dihydrochrysene (chrysene-1,2-dihydrodiol) and *anti*-chrysene-1,2-diol-3,4-epoxide enantiomers in mouse skin (according to [79])

Compound	Dose (μmol)	% of mice with tumors	No. of tumors per mouse
(+)-Chrysene-1,2-dihydrodiol	0.4	3	0.03
	1.2	23	0.40
(−)-Chrysene-1,2-dihydrodiol	0.4	67	1.47
	1.2	83	2.77
(+)-*anti*-Chrysene-1,2-diol-3,4-epoxide	0.4	31	0.52
	1.2	60	1.47
(−)-*anti*-Chrysene-1,2-diol-3,4-epoxide	0.4	10	0.13
	1.2	23	0.33
(±)-*anti*-Chrysene-1,2-diol-3,4-epoxide	1.2	53	1.33

Table 13 Chiral pulmonary and hepatic carcinogenicity of *trans*-1,2-dihydroxy-1,2-dihydrochrysene (chrysene-1,2-dihydrodiol) and *syn*- and *anti*-chrysene-1,2-diol-3,4-epoxide enantiomers in newborn mice (according to [79])

Compound	Dose (μmol)	Pulmonary tumor		Hepatic tumor	
		% of mice with tumors	No. of tumor per mouse	% of mice with tumors	No. of tumors per mouse
(+)-Chrysene-1,	1.4[a]	20	0.22	0	0
2-dihydrodiol	1.4[b]	16	0.54	16	0.22
(−)-Chrysene-1,	1.4[a]	89	10.62	0	0
2-dihydrodiol	1.4[b]	95	7.41	57	2.3
(±)-Chrysene-1,	1.4[a]	89	3.78	0	0
2-dihydrodiol	1.4[b]	84	3.32	26	0.68
(+)-*syn*-Chrysene-1,	0.7[a]	21	0.21	0	0
2-diol-3,4-epoxide	0.7[b]	18	0.20	3	0.03
(−)-*syn*-Chrysene-1,	0.7[a]	27	0.27	0	0
2-diol-3,4-epoxide	0.7[b]	16	0.20	0	0
(+)-*anti*-Chrysene-1,	0.7[a]	88	6.59	0	0
2-diol-3,4-epoxide	0.7[b]	91	4.34	23	0.37
(−)-*anti*-Chrysene-1,	0.7[a]	8	0.08	0	0
2-diol-3,4-epoxide	0.7[b]	14	0.16	0	0

[a]Female and male[b] mice, respectively

epoxide was the most mutagenic diol-epoxide and about 5–40 times more active than the other three optical isomers. Furthermore, the same group studied the enantioselective toxicities of *trans*-1,2-dihydroxy-1,2-dihydrochrysene (chrysene-1,2-dihydrodiol) and chrysene-1,2-diol-3,4-epoxides in two different mouse tumor models [79]. In the animals, the skin, pulmonary, and hepatic carcinogenicity of these chiral pollutants was investigated. Skin carcinogenicity is presented in Table 12. Table 13 summarizes the extent of pulmonary and hepatic tumor formation in newborn mice.

Table 12 shows that only 3% tumors were found when (+)-enantiomer of chrysene-1,2-dihydrodiol was injected, while 67% carcinogenicity was observed with the (−)-enantiomer. Contrarily, the tumor-initiating activity of (+)-, (−)-, and (±)-chrysene-1,2-diol-3,4-epoxides (at a dose of 1.2 μmol each) was 21%, 13%, and 25%,

respectively. Again, the toxicity of the racemic mixture was greater than the effects seen for (−)- and (+)-enantiomers, which might be due to the interference of both enantiomers with each other. A perusal of Table 13 indicates that again the (−)-*trans*-chrysene-1,2-dihydrodiol is more toxic than its (+)-antipode in both male and female mice.

Benz[*a*]anthracene(BA)-3,4-diol-1,2-epoxide (BADE) results from regio- and stereoselective metabolism of BA. The tumorigenic activities of the (+)- and (−)-enantiomers of *trans*-3,4-dihydroxy-3,4-dihydrobenz[*a*]anthracene (BA-3,4-dihydrodiol) and racemic diastereomers of BADE were studied in newborn Swiss-Webster mice [80]. Furthermore, Tang *et al.* determined the tumorigenic potencies of racemic *syn*- and *anti*-7,12-dimethylbenz[*a*]anthracene(DMBA)-3,4-diol-1,2-epoxides (DMBADE) via the two-stage initiation–promotion protocol in mouse skin [81]. They observed that both *syn*- and *anti*-DMBADE were active tumor initiators and that the occurrence of papillomas was dependent on the dose of carcinogen applied.

Enantioselective Toxicities of Algae Toxins

Tetrodotoxin and saxitoxin are major marine toxic chiral pollutants. The toxic effects of saxitoxin have been observed in some part of the world, and, hence, sometimes filter-feeding shell fish industries have been affected. It has also been reported that the marine environment is rich with (−)-enantiomer of saxitoxin. Other chiral neurotoxins (anatoxin, homoanatoxin, *etc.*) are produced in the aquatic environment as well [82]. Therefore, sometimes, water becomes toxic due to the presence of these compounds, and several reports have been published on the death of cattle and dogs due to intoxication [83, 84]. Accordingly, the presence of these toxic chiral pollutants may be health threatening for humans as well. Unfortunately, no reports have been published on the enantioselective toxicities of these toxins yet.

Enantioselective Toxicities of Drugs and Pharmaceuticals

A myriad of different drugs are being used by people, and among them a great part is chiral in nature. Therefore, the presence of such types of drug enantiomers in the environment may be problematic and hazardous. Weigel [14] reported on the presence of several drugs in aquatic environments at high concentrations. Kümmerer [85] reported the presence of several drugs in surface, ground, and drinking water. In 1960, thalidomide [(*R,S*)-*N*-(2,6-dioxo-3-piperidyl) phthalimide] was introduced as a sedative drug in Europe, and, unfortunately, teratogenic effects of this drug occurred in embryos due to the highly toxic *S*-enantiomer [86]. Ifosfamide is a cyclophosphamide analog, which possesses toxicity that is enantioselective in nature. Masurel *et al.* [87] studied the enantioselective toxicity of this drug in rats. The authors injected the racemate and the enantiomers separately into nontumor-

bearing rats at doses of 550–650 mg/kg. The mean weight loss (at highest dose) was 30%, 20%, and 17% for the (+)- and (−)-enantiomers and the racemic mixture of ifosfamide, respectively. Furthermore, the authors observed signs of acute bladder toxicity, as blood was reported in the urine of rats when (−)-ifosfamide was injected. Similarly, there are several other drugs whose enantiomers are selectively toxic. L-DOPA has long been introduced for the treatment of Parkinson's disease, albeit D-DOPA turned out to be toxic [88, 89]. In 1986, Domino [90] described enantioselective opioid hallucinogen interactions of *N*,*N*-dimethyltryptamine and lysergic acid *N*,*N*-diethylamide in rats.

Conclusions

It is clear from this chapter that the chirality plays an important role in environmental toxicology affecting our lives. The chiral pollutants are widely distributed. Enantioselective toxicities have been reported for several xenobiotics in the earth ecosystem. In spite of this, scientists have only rarely been attracted toward this problem, and only few groups are addressing this issue. Therefore, there is still an urgent need to more comprehensively explore the enantioselective toxicities of chiral pollutants. The existing toxicological data of pollutants, mostly pertaining to their racemic forms, must be refined in terms of enantioselective toxicities. Even achiral pollutants are sometimes metabolized into chiral follow-up products, and, therefore, the study of these chiral species is a demanding field. Analysis of the specific toxicities of the chiral pollutants is essential and may be useful for controlling certain adverse health effects and diseases. In summary, the role of chirality in environmental toxicology is a burning area and needs more attention of the world's scientists for the welfare of human beings.

References

1. TSCA (1984) Toxic substance control act. US-EPA-III, 344-348. Online available at http://www.epa.gov/regulations/laws/tsca.html
2. Hutson DH, Roberts TR (1990) Environmental fate of pesticides, vol 7. Wiley, New York
3. Ali I, Jain CK (1998) Ground water contamination and health hazards by some of the most commonly used pesticides. Curr Sci 75:1011–1014
4. Ali I, Aboul-Enein HY (2004) Chiral pollutants: distribution, toxicity and analysis by chromatography and capillary electrophoresis. Wiley, Chichester
5. Kelvin L, Kallenborn R, Hühnerfuss H (2000) Chiral environmental pollutants: trace analysis and ecotoxicology. Springer, Berlin
6. Hegstrom R, Kondepudi DK (1990) The handedness of the universe. Sci Am 262:108–115
7. Kondepudi D, Durand DJ (2001) Chiral asymmetry in spiral galaxies? Chirality 13:351–356
8. Bailey J, Chrysostomou A, Hough JH, Gledhill TM, McCall A, Clark S, Menard F, Tamura M (1998) Circular polarization in star-formation regions: implications for biomolecular homochirality. Science 281:672–674

9. Haüy RJ (1809) Tableau Comparatif des Résultats de Cristallographie, et de l'Analyse Chimique Relativement á la Classification des Minéraux. p XVII, Courcier Paris, France
10. Pasteur L (1848) Mémoire sur la relation qui peut exister entre la forme cristalline et la composition chimique, et sur la cause de la polarisation rotatoire. Comptes Rendus, Acad Sci (Paris) 26:535–538
11. Lewis DL, Garrison AW, Wommack KE, Whittemore A, Steudler P, Melillo J (1999) Influence of environmental changes on degradation of chiral pollutants in soils. Nature 401:898–901
12. Ariens EJ, van Rensen JJS, Welling W (1988) Stereoselectivity of pesticides, biological and chemical problems, chemicals in agriculture, vol 1. Elsevier, Amsterdam
13. Buser HR, Müller MD, Rappe C (1992) Enantioselective determination of chlordanes component using chiral high-resolution gas chromatography mass-spectrometry with application to environmental samples. Environ Sci Technol 26:1533–1540
14. Weigel S (1998) Entwicklung einer Methode zur Extraktion Organischer Spurenstoffe aus groβvolumigen Wasserproben mittels Festphasen. Master Thesis (Diplomarbeit), University of Hamburg, Germany, p 86
15. Buser HR, Poiger T, Müller MD (1999) Occurrence and environmental behaviour of the chiral pharmaceutical drug ibuprofen in surface water and waste water. Environ Sci Technol 33: 2529–2535
16. Ali I, Aboul-Enein HY, Kümmerer K (2009) Analyses of drugs and pharmaceuticals in the environment. In: Xing B, Senesi N, Huang PM (eds) Biophysico-chemical processes of anthropogenic organic compounds in environmental systems, IUPAC sponsored book series, vol 3. Wiley, New York
17. Vetter W, Bartha R, Stern G, Tomy G (1998) Enantioselective determination of two major compounds of technical toxaphene in Canadian lake sediment cores from the last 60 years. Organohalog Compds 35:343–346
18. Vetter W, Bartha R, Stern G, Tomy G (1999) Enantioselective determination of two persistent chlorobornane congeners in sediment from a toxaphene-treated Yukon lake. Environ Toxicol Chem 18:2775–2781
19. Rappe C, Haglund P, Buser HR, Müller MD (1997) Enantioselective determination of chiral chlorobornanes in sediments from the Baltic Sea. Organohalog Compds 31:233–237
20. Benicka E, Novakovsky R, Hrouzek J, Krupcik J, Sandra P, Zeeuw JD (1996) Multidimensional gas chromatographic separation of selected PCB atropisomers in technical formulations and sediments. J High Resolut Chromatogr 19:95–98
21. Wong CS, Garrisson AW, Foreman WT (2001) Enantiomeric composition of chiral polychlorinated biphenyl atropisomers in aquatic bed sediment. Environ Sci Technol 35:33–39
22. Biselli S, Dittmer H, Gatermann R, Kallenborn R, König WA, Hühnerfuss H (1999) Separation of HHCB, AHTN, ATII, AHDI and DPMI by enantioselective capillary gas chromatography and preparative separation of HHCB and ATII by enantioselective HPLC. Organohalog Compds 40:599–602
23. Moisey J, Fisk AT, Hobson KA, Norstrom RJ (2001) Hexachlorocyclohexane (HCH) isomers and chiral signatures of α-HCH in the arctic marine food web of the northwater polynya. Environ Sci Technol 35:1920–1927
24. Aigner E, Leone A, Falconer R (1998) Concentration and enantiomeric ratio of organochlorine pesticides in soils from the US corn belt. Environ Sci Technol 32:1162–1168
25. Wiberg K, Harner T, Wideman JL, Bidleman TF (2001) Chiral analysis of organochlorine pesticides in Alabama soils. Chemosphere 45:843–848
26. White JC, Mattina MJI, Eitzer BD, Berger WI (2002) Tracking chlordane compositional and chiral profiles in soil and vegetation. Chemosphere 47:639–646
27. Ridal JJ, Bidleman TF, Kerman BR, Fox ME, Strachan WMJ (1997) Enantiomers of α-hexachlorocyclohexane as tracers of air-water gas exchange in lake Ontario. Environ Sci Technol 31: 1940–1945
28. Ulrich EM, Hites RA (1998) Enantiomeric ratios of chlordane-related compounds in air near the great lakes. Environ Sci Technol 32:1870–1874

29. Bidleman TF, Jantunen LM, Wiberg K, Harner T, Brice K, Su K, Falconer RL, Lenone AD, Aigner EJ, Parkhurst W (1998) Soil as a source of atmospheric heptachlor epoxide. Environ Sci Technol 32:1546–1548
30. Wiberg K, Jantunen LM, Harner T, Wideman JL, Bidleman TF, Brice K, Su K, Falconer RL, Leone AD, Parkhurst W, Alegria H (1997) Chlordane enantiomers as source markers in ambient air. Organohalog Compds 33:209–213
31. Buser HR, Müller MD (1993) Enantioselective determination of chlordane components, metabolites and photoconversion products in environmental samples using chiral high-resolution gas chromatography and mass spectrometry. Environ Sci Technol 27:1211–1220
32. Möller K, Bretzke C, Hühnerfuss H, Kallenborn R, Kinkel JN, Kopf J, Rimkus G (1994) The absolute configuration of (+)-α-1,2,3,4,5,6-hexachlorocyclohexane, and its permeation through the seal blood-brain. Angew Chem Int Ed 33:882–884
33. König WA, Icheln D, Runge T, Pfaffenberger B, Ludwig P, Hühnerfuss H (1991) Gas chromatographic enantiomer separation of agrochemicals using modified cyclodextrins. J High Resolut Chromatogr 14:530–536
34. Wiberg K, Letcher R, Sandau C, Norstrom R, Tysklind M, Bidleman T (1998) Enantioselective analysis of organochlorines in the arctic marine food chain: Chiral biomagnifications factors and relationship of enantiomeric ratios, chemical residues and biological data. Organohalog Compds 35:371–374
35. Müller MD, Schlabach M, Oehme M (1992) Fast and precise determination of α-hexachlorocyclohexane enantiomers in environmental samples using chiral high-resolution gas chromatography. Environ Sci Technol 26:566–569
36. Wong CS, Garrisson AW, Smith PD, Foreman WT (2001) Enantiomeric composition of chiral polychlorinated biphenyls atropisomers in aquatic and Riparian biota. Environ Sci Technol 35:2448–2454
37. Fisk AT, Moisey J, Hobson KA, Karnovsky NJ, Norstrom RJ (2001) Chlordane components and metabolites in seven species of Arctic seabirds from the northwater polynya: relationships with stable isotopes of nitrogen and enantiomeric fractions of chiral components. Environ Pollut 113:225–238
38. Möller K, Hühnerfuss H, Rimkus G (1993) On the diversity of enzymatic degradation pathways of α-hexachlorocyclohexane as determined by chiral gas chromatography. J High Resolut Chromatogr 16:672–673
39. Pfaffenberger B, Hardt I, Hühnerfuss H, König WA, Rimkus G, Glausch A, Schurig V, Hahn J (1994) Enantioselective degradation of α-hexachlorocyclohexane and cyclodiene insecticides in Roe-deer liver samples from different regions of Germany. Chemosphere 29:1543–1554
40. Alder L, Palavinskas R, Andrews P (1996) Enantioselective determination of toxaphene components in fish, monkey adipose tissue from a feeding study and human milk. Organohalog Compds 28:410–415
41. Möller K, Hühnerfuss H, Wölfle D (1996) Differential effects of the enantiomers of α-hexachlorocyclohexane (α-HCH) on cytotoxicity and growth stimulation in primary rat hepatocytes. Organohalog Compds 29:357–360
42. Miyazaki A, Hotta T, Marumo S, Sakai M (1978) Synthesis, absolute chemistry, and biological activity of optically active cyclodiene insecticides. J Agric Food Chem 26:975–977
43. Miyazaki A, Sakai M, Marumo S (1980) Synthesis and biological activity of optically active heptachlor, 2-chloroheptachlor, and 3-chloroheptachlor. J Agric Food Chem 28:1310–1311
44. McBlain WA, Lewin V (1976) Differing estrogenic activities for the enantiomers of o, p′-DDT in immature female rats. Can J Physiol Pharmacol 54:629–632
45. Hoekstra PF, Burnison BK, Neheli T, Muir DCG (2001) Enantiomer-specific activity of o, p′-DDT with the human estrogen receptor. Toxicol Lett 125:75–81
46. Miyazaki A, Nakamura T, Marumo S (1989) Stereoselectivity in metabolic sulfoxidation of propaphos and biological activity of chiral propaphos sulfoxide. Pestic Biochem Physiol 33:11–15
47. Miyazaki A, Nakamura T, Kawaradani M, Marumo S (1988) Resolution and biological activity of both enantiomers of methamidophos and acephate. J Agric Food Chem 36:835–837

48. Lang D, Criegee D, Grothusen A, Saalfrank RW, Böcker RH (1996) *In vitro* metabolism of atrazine, terbuthylazine, ametryne, and terbutryne in rats, pigs, and humans. Drug Metab Dispos 24:859–865
49. De Jong LPA, Benschop HP (1991) Interactions of esterases with soman and other chiral anticholinesterase organophosphates. In: Cholinesterases, Proceedings of the 3rd International Meeting on Cholinesterases, Conference Proceedings Series, American Chemical Society, Washington DC, p 240
50. Rodriguez OP, Muth GW, Berkman CE, Kim K, Thompson CM (1997) Inhibition of various cholinesterases with the enantiomers of malaoxon. Bull Environ Contam Toxicol 58:171–176
51. Gonzalez MJ, Fernandez MA, Hernandez LM (1991) Level of chlorinated insecticides, PCBs and PCB congeners in Spanish gull eggs. Arch Environ Contam Toxicol 20:343–348
52. König WA, Gehrcke B, Runge T, Wolf C (1993) Gas chromatographic separation of atropisomeric alkylated and polychlorinated biphenyls using modified cyclodextrins. J High Resolut Chromatogr 16:376–378
53. Ahlborg UG, Becking GC, Birnbaum LS, Brouwer A, Derks HJGM, Feeley M, Golor G, Hanberg A, Larsen JC, Liem AKD, Safe SH, Schlatter C, Waern F, Younes M, Yränheikki E (1994) Toxic equivalency factors for dioxin-like PCBs: Report on WHO-ECEH and IPCS consultation, December 1993. Chemosphere 28:1049–1067
54. Püttmann M, Mannschreck A, Oesch F, Robertson LW (1989) Chiral effects in the induction of drug-metabolizing enzymes using synthetic atropisomers of polychlorinated biphenyls (PCBs). Biochem Pharmacol 38:1345–1352
55. Rodman EL, Shedlofsky SI, Mannschreck A, Püttmann M, Swim AT, Robertson LW (1991) Differential potency of atropisomers of polychlorinated biphenyls on cytochrome P450 induction and uroporphyrin accumulation in the chick embryo hepatocyte culture. Biochem Pharmacol 41:915–922
56. Bakke J, Gustafsson JA (1984) Mercapturic acid pathway metabolites of xenobiotics: generation of potentially toxic metabolites during enterohepatic circulation. Trends Pharmacol Sci 5:517–521
57. Lund OB, Orberg J, Bergman A, Larsson C, Bäcklin BM, Hakansson H, Madej A, Brouwer A, Brunström B (1999) Chronic and reproductive toxicity of a mixture of 15 methylsulfonyl-polychlorinated biphenyls and 3-methylsulfonyl-2,2-*bis*-(4-chlorophenyl)-1,1-dichloroethene in mink (*Mustela vison*). Environ Toxicol Chem 18:292–298
58. Nakanishi Y, Shigematsu N, Kurita Y, Matsuba K, Kanegae H, Ishimaru S, Kawazoe Y (1985) Respiratory involvement and immune status in yusho patients. Environ Health Perspect 59: 31–36
59. Ellerichmann T, Bergman A, Franke S, Hühnerfuss H, Jakobsson E, König WA, Larsson C (1998) Gas chromatographic enantiomer separation of chiral PCB methyl sulfons and identification of selectively retained enantiomers in human liver. Fresenius Environ Bull 7:244–257
60. Levin W, Wood AW, Chang RL, Slaga TJ, Yagi H, Jerina DM, Conney AH (1977) Marked differences in the tumor-initiating activity of optically pure (+)- and (–)-*trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene on mouse skin. Cancer Res 37:2721–2725
61. Borgen A, Darvey H, Castagnoli N, Crocker TT, Rasmussen R, Wang IW (1973) Metabolic conversion of benzo[*a*]pyrene by Syrian hamster liver microsomes and binding of metabolites to deoxyribonucleic acid. J Med Chem 16:502–506
62. Daudel P, Duquesne M, Vigny P, Grover PL, Sims P (1975) Fluorescence spectral evidence that benzo[*a*]pyrene-DNA products in mouse skin arise from diol-epoxides. Fed Eur Biochem Soc Lett 57:250–253
63. Jerina DM, Daly JW (1974) Arene oxides: a new aspect of drug metabolism. Science 185: 573–582
64. Miller EC, Miller JA (1974) Biochemical mechanism of chemical carcinogenesis. In: Busch H (ed) Molecular biology of cancer. Academic, New York, NY
65. Sims P, Grover PL (1974) Epoxides in polycyclic aromatic hydrocarbon metabolism and carcinogenesis. Adv Cancer Res 20:165–274

66. Sims P, Grover PL, Swaisland A, Pal K, Hewer A (1974) Metabolic activation of benzo[*a*]-pyrene proceeds by a diol-epoxide. Nature 252:326–328
67. Yagi H, Hernandez O, Jerina DM (1975) Synthesis of (±)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10- tetrahydrobenzo[*a*]pyrene, a potential metabolite of the carcinogen benzo[*a*]pyrene with stereochemistry related to the antileukemic triptolides. J Am Chem Soc 97:6881–6883
68. Kapitulnik J, Lewin W, Conney AH, Yagi H, Jerina DM (1977) Benzo[*a*]pyrene 7,8-dihydrodiol is more carcinogenic than benzo[*a*]pyrene in newborn mice. Nature 266:378–380
69. Kapitulnik J, Wislocki PG, Lewin W, Yagi H, Jerina DM, Conney AH (1978) Tumorigenicity studies with diol-epoxides of benzo[*a*]pyrene which indicate that (±)-*trans*-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene is an ultimate carcinogen in newborn mice. Cancer Res 38:354–358
70. NAS (1972) Committee on the biological effects of atmospheric pollutants, particulate polycyclic organic matter. National Academy of Science, Washington DC
71. Newbold RF, Brookes P (1976) Exceptional mutagenicity of a benzo[*a*]pyrene diol epoxide in cultured mammalian cells. Nature 261:52–54
72. Wislocki PG, Wood AW, Chang RL, Lewin W, Yagi H, Hernandez O, Jerina DM, Conney AH (1976) High mutagenicity and toxicity of a diol epoxide derived from benzo[*a*]pyrene. Biochem Biophys Res Commun 68:1006–1012
73. Wood AW, Wislocki PG, Chang RL, Lewin W, Lu AYH, Yagi H, Hernandez O, Jerina DM, Conney AH (1976) Mutagenicity and cytotoxicity of benzo[*a*]pyrene benzo-ring epoxides. Cancer Res 36:3358–3366
74. Wood AW, Chang RL, Levin W, Yagi H, Thakker DR, Jerina DM, Conney AH (1977) Differences in mutagenicity of the optical enantiomers of the diastereomeric benzo[*a*]pyrene 7,8-diol-9,10-epoxides. Biochem Biophys Res Commun 77:1389–1396
75. Slaga TJ, Bracken WJ, Gleason G, Levin W, Yagi H, Jerina DM, Conney AH (1979) Marked differences in the skin tumor-initiating activities of the optical enantiomers of the diastereomeric benzo[*a*]pyrene 7,8-diol-9,10-epoxides. Cancer Res 39:67–71
76. Chang RL, Wood AW, Lewin W, Mah HD, Thakker DR, Jerina DM, Conney AH (1979) Differences in mutagenicity and cytotoxicity of (+)- and (–)-benzo[*a*]pyrene 4,5-oxide: a synergistic interaction of enantiomers. Proc Natl Acad Sci USA 76:4280–4284
77. Levin W, Buening MK, Wood AW, Chang RL, Kedzierski B, Thakker DR, Boyd DR, Gadaginamath GS, Armstrong RN, Yagi H, Karle JM, Slaga TJ, Jerina DM, Conney AH (1980) An enantiomeric interaction in the metabolism and tumorigenicity of (+)- and (–)-benzo[*a*]pyrene 7,8-oxide. J Biol Chem 255:9067–9074
78. Wood AW, Chang RL, Levin W, Yagi H, Tada M, Vyas KP, Jerina DM, Conney AH (1982) Mutagenicity of the optical isomers of the diastereomeric bay-region chrysene 1,2-diol-3,4-epoxides in bacterial and mammalian cells. Cancer Res 42:2972–2976
79. Chang RL, Levin W, Wood AW, Yagi H, Tada M, Vyas KP, Jerina DM, Conney AH (1983) Tumorigenicity of enantiomers of chrysene 1,2-dihydrodiol and of the diastereomeric bay-region chrysene 1,2-diol-3,4-epoxides on mouse skin and in newborn mice. Cancer Res 43:192–196
80. Wislocki PG, Buening MK, Levin W, Lehr RW, Thakker DR, Jerina DM, Conney AH (1979) Tumorigenicity of the diastereomeric benz[*a*]anthracene 3,4-diol-1,2-epoxides and the (+)- and (–)-enantiomers of benz[*a*]anthracene 3,4-dihydrodiol in newborn mice. J Natl Cancer Inst 63:201–204
81. Tang MS, Vulimiri SV, Viaje A, Chen JX, Bilolikar DS, Morris RJ, Harvey RG, Slaga TJ, DiGiovanni J (2000) Both (±)*syn*- and (±)*anti*-7,12-dimethylbenz[*a*]anthracene-3,4-diol-1,2-epoxides initiate tumors in mouse skin that possess -CAA- to -CTA- mutations at codon 61 of c-H-*ras*. Cancer Res 60:5688–5695
82. Carmichael WW (1981) Fresh water blue green algae (cyanobacteria) in the water environment—algal toxins and health. In: Carmichael WW (ed) The water environment, algal toxins and health. Plenum, New York, NY
83. Skulberg OM, Codd GA, Carmicheal WW (1984) Toxic blue-green algal bloom in Europe: a grazing problem. Ambio 13:244–247

84. Haugen JE, Skulberg OM, Andersen RA, Alexander J, Lilleheil G, Gallagher T, Brough PA (1994) Rapid analysis of cyanophyte neurotoxins: an improved method for quantitative analysis of anatoxin-a and homoanatoxin-a in the sub-ppb to ppb range. Algae Stud 75: 111–121
85. Kümmerer K (2001) Drugs in the environment: emission of drugs, diagnostic aids and disinfectants into wastewater by hospital in relation to other sources—a review. Chemosphere 45:957–969
86. Annas GJ, Elias S (1999) Thalidomide and the Titanic: reconstructing the technology tragedies of the twentieth century. Am J Public Health 89:98–101
87. Masurel D, Houghton PJ, Young CL, Wainer IW (1990) Efficacy, toxicity, pharmacokinetics, and *in vitro* metabolism of the enantiomers of ifosfamide in mice. Cancer Res 50:252–255
88. Cotzias GC, Papvasilou PS, Gellene R (1969) Modification of Parkinsonism—chronic treatment with L-DOPA. N Engl J Med 280:337–345
89. Poskanzer DC (1969) L-Dopa in Parkinson's syndrome. N Engl J Med 280:382–383
90. Domino EF (1986) Opioid-hallucinogen interactions. Pharmacol Biochem Behav 24:401–405

Genetic Variability in Molecular Responses to Chemical Exposure

Chirag J. Patel and Mark R. Cullen

Abstract Individuals differ in their response to environmental exposures. In the following, we describe examples and paradigms of studying heritable differences in response to exposure—commonly known as "gene–environment interaction" or "ecogenetics"—and their relation to disease etiology and susceptibility. Our discussion is framed in three parts. In the first, we describe replicated examples of studies that have typified the field, single genetic variant, and exposure associations to disease. Second, we describe how studies have scaled up search for interaction using genome-wide measurement modalities, bioinformatics, and model organisms. Finally, we discuss a more comprehensive representation of chemical exposures as the "envirome" and how we may use the envirome to examine interplay between genetics and the environment.

Keywords Gene–environment interactions · Pharmacogenetics · Ecogenetics · Single nucleotide polymorphism · (Non-)synonymous variant · Phase I/II biotransformation · Paraoxonase · Epigenetic variant · Genome-wide association study · Gene–environment-wide interaction study · Environment-wide association study · Comparative Toxicogenomics Database · Systems genetics · Envirome · Toxome

Introduction

Host response to chemical exposures is not only dependent on compound type and dosage but variability of the host itself. The heritable code passed on from generation to generation is known amorphously as genetics and is recorded latently in

C.J. Patel and M.R. Cullen (✉)
Department of Medicine, Stanford University, 1265 Welch Road, X338, Stanford, CA 94305, USA
e-mail: cjp@stanford.edu; mrcullen@stanford.edu

A. Luch (ed.), *Molecular, Clinical and Environmental Toxicology*,
Experientia Supplementum 101, DOI 10.1007/978-3-7643-8340-4_15,

DNA as units called genes. Genes, in turn, carry instructions for functional units, message RNA, and proteins. Evolutionarily speaking, difference in response coded by genetics is the "substrate" of natural selection. That is, as new challenges, such as chemical exposures, arise, genetic variability determines an individual's survivability and ability pass on their code to subsequent generations. More practically, we may think of "survivability" in the context of disease, considering how the environments in which we live modify our innate genetic risk for disease: in other words, considering "gene–environment" interactions. Specifically, gene–environment interactions can be thought of as phenotypic variability attributed to the joint effect of genetic variation and environmental exposures that presumably cannot be described with either genetics or exposures alone.

For example, take the disease phenotype phenylketonuria (PKU) [1]. Those with PKU have inherited a rare genetic variant that codes for a deficient phenylalanine hydroxylase liver enzyme and are unable to convert the amino acid compound phenylalanine from their diets to another amino acid, tyrosine. In the presence of both the deficient enzyme and phenylalanine, an intermediate compound accumulates, phenylketone, leading to mental retardation. However, even with the rare genetic variant coding for the deficient protein, controlling phenylalanine exposure mitigates adverse phenotypes.

The study of gene–environment interactions is akin to "pharmacogenetics," which relates genetic differences and variability of molecular responses to drugs. In fact, the term "ecogenetics"—the study of gene–environment interactions—discriminates environmental responses from drug responses. Over eight decades ago, Archibald Garrod undertook studies regarding genetics and metabolic-focused response to environmental chemicals, observing "inborn errors of metabolism" in adverse phenotypes such as alkaptonuria [2]. In 1931, Garrod further described adverse phenotypes that occur only in certain individuals, ". . .substances contained in particular foods, certain drugs, and exhalations of animals or plants *produce in some people effects wholly out of proportion to any which they bring about in average individuals* (sic)" [3]. This observation was the first classic pharmacogenetic "responder" *vs*. "nonresponder" phenotype that would come to dominate the field. Later, Motulsky set the stage for pharmacogenetics (and later ecogenetics) in which he described the adverse response to drugs as an environmental and dose-dependent "trigger" for genetically susceptible individuals [4].

What is a "genetic variant" and polymorphism? At its highest conceptual level, most heritable differences that vary from individual to individual can be attributed to genetic variants. Genetic variants most commonly reside in DNA as (1) single nucleotide polymorphisms (SNPs), inherited in blocks called "haplotypes" [5]; (2) segments that are added, deleted, and or inverted and vary in copy number called structural variation [6]; and (3) repeated segments vary in their repeat number such as microsatellites [7]. The locations along the genome where these variants occur are called "loci," and their alternate states are called "alleles." Most DNA variants occur as SNPs. As of this writing, there are about 20 million of these catalogued in the National Centers for Biotechnology Information (NCBI) dbSNP database [8] and will comprise bulk of the examples illustrated below. Due to

linkage disequilibrium, or nonindependent association of alleles at distinct loci, alleles are inherited as sets called haplotypes which themselves may be considered a type of genetic variant. When a variant reaches 1% frequency in the population, it is called a "polymorphism." Often polymorphic loci induce change in function when they exist in either coding regions or regulatory regions of the genome. For example, a locus within a coding region might cause an amino acid change (called a "non-synonymous" variant). On the other hand, due to redundancy in the mechanism translating the gene to protein, they need not lead to an amino acid change (called a "non-synonymous" variant). However, while non-synonymous variants may directly lead to altered protein function, both types of variants are important to examine. For example, variants may lie within regulatory regions, consequently influencing the machinery by which a gene is expressed as RNA and protein.

There are other types of physical "heritable" variants, epigenetic variants, that are not coded within one's DNA [9]. Epigenetics is the study of heritable variation in gene expression that is coded outside of DNA. There are two mechanisms by which epigenetic variability is coded: one is a known as methylation of DNA and the second is histone modification. In methylation, cytosine- and guanine-rich DNA segments are chemically modified through addition of methyl groups. Histones, the protein complex that package DNA, may also be chemically modified via methylation, acetylation, phosphorylation, and glycosylation, among others. The prototypical example of epigenetic process comes with cellular differentiation during early development of the embryo. In early development, germ cells and the zygote undergo epigenetic reprogramming, and methylation patterns determine cell lineage and fate [10, 11]. Furthermore, epigenetic mechanisms can change gene expression under exogenous influence, theoretically allowing for environmental heritability. However, the extent of their "Lamarckian heritability" imposed by a lifetime of environmental influence is debatable [12, 13]. While investigators have shown epigenetic state as "biomarkers" or surrogates of exposure, associated to disease phenotypes such as in cancer tumors [14–18], epigenetic-environment chemical interactions have not been shown [13, 19].

Types of phenotypic responses under consideration here are no different from those examined in pharmacology or toxicology. For example, genetic variants in the presence of environmental exposures interest us both in terms of kinetics, or how a genetic variant leads to change in chemical metabolism, how a genetic variant may change physiological target responses such as altered gene expression or protein activity.

The interaction of human genetic variants and chemical exposures is measured and assessed through population or epidemiological association, in which the presence of both a genetic variant and chemical factor is associated with a disease phenotype [20, 21]. In statistical models, the hypothesis of joint effect is tested against the marginal association between each of the factors alone and phenotype. However, as both epidemiologists and toxicologists alike would note, population-based statistical interaction does not easily map onto or prove to biological or molecular interaction [22, 23].

We divide our exploration of the interaction between genetic variants and chemical exposures into three parts. In the first, we take examples from the established paradigm to illustrate how variants in single genes interact with single chemical exposures, focusing on toxicokinetic responses. In the second part, we describe how studies have been framed to scale up the search for genetic variants across the entire genome through genome-wide association studies (GWAS) and systems genetics. We also will discuss how interaction-associated phenotypes may be predicted applying bioinformatics tools using publicly available data. In the final part, we introduce a third generation of studies to scale up both the search for interacting genes and chemical factors using what we call the "envirome." Taking a cue from Garrod, our examples and discussion shall be centered on molecular responses in the context of disease and disease-related phenotypes.

Single-Gene, Single-Chemical-Based Interactions

Genetic Variants Influencing the Metabolic Transformation of Chemicals

The beginning of the study of genetic differences in response to chemical exposure focused on mendelian differences in metabolism in the context of adverse phenotypes, namely, disease. Indeed, these studies preceded the abstract definition of a "gene," in which Archibald Garrod described inherited differences in metabolism that led to diseases such as alkaptonuria [2]. For example, alkaptonuria arises from defects in a gene coding for the metabolic enzyme, homogentisate 1,2-dioxygenase, which participates in the degradation of tyrosine. These defects lead to the accumulation of a by-product of tyrosine, homogenetic acid, which in turn damages cartilage [24]. Presciently, Garrod showed how "proteid" intake was in fact related to signs of the disease-given family history.

Since then, most studies have directly aimed to observe molecular response in the context of metabolic, or "toxicokinetic" genetic differences in phase I and II enzymes. In general, toxicokinetic response involves transforming a chemical to facilitate its digestion and excretion. In some instances, however, the transformation can lead to a more damaging chemical species, analogous to Garrod's original finding. Phase I reactions are known to be, for the most part, oxidative, in which different functional groups (i.e., –OH, $–NH_2$, –SH) are introduced to the compound to increase solubility for excretion. In phase II, the product of a phase I reaction go through reactive processes such as acetylation or conjugation with glutathione, again to increase solubility. We begin by summarizing the work characterizing genetic differences in these phase I and II enzymes, specifically in the context of disease.

Phase I Biotransformation Enzymes

The phase I biotransformation enzymes are involved in the oxidation of chemicals and include the cytochrome P450-dependent monooxygenase (CYP) class of enzymes, the most widely studied transformation enzymes of all, and the flavin-dependent monooxygenases (FMOs). Nearly all chemicals undergo phase I transformation, particularly through the CYP family of enzymes. The CYP family is quite large as there are 57 different enzymes encoded in the human genome; bioinformatics database resources have been devoted to cataloging their mutations and nomenclature [25]. Many mutations in both the CYP and FMO families are considered to be polymorphic (greater than 1% allele frequency).

The CYP enzyme CYP1A1, also known as aryl hydrocarbon hydroxylase (AHH), is known to oxidize or activate environmental compounds such as polycyclic aromatic hydrocarbons (PAHs), including benzo[*a*]pyrene [26] and estrogens [27]. Early hypotheses regarding the effect of different CYP variants on the metabolic efficiency of PAHs from cigarette smoke exposure came in the context of lung cancer risk [28] and altered function due to some of the variants. One such study showed a race-dependent self-regulation of CYP1A1 gene expression as a function of a genotype found in exon 7 of the gene [29].

Phase II Biotransformation Enzymes

Whereas phase I enzymes are dominated by the CYP and FMO enzymes focused on the addition of four functional groups, enzymes classified as phase II are involved in the larger variety of processes, such as glucuronidation, sulfation, acetylation, methylation, and conjugation of glutathione. Molecular processes involving acetylation carried out by the *N*-acetyltransferases (NATs) and associated proteins and conjugation with glutathione, performed by the glutathione-*S*-transferases (GSTs), have received the most attention in relation to variable host responses.

NATs consist of two genes (NAT1, NAT2) and are enzymes that "activate" (*N*-acetylation) and "deactivate" (*O*-acetylation) heterocyclic amine-bearing compounds and require the cofactor acetyl coenzyme for the acetyl group. Like the CYP class of genes, the NAT genes are highly polymorphic and result in different phenotype frequencies associated with ethnicity. For example, half of the European population and a majority of Africans are known to be "slow" acetylators, but only a minority of those of Asian ancestry are slow acetylators.

Altered function due to NAT variants and exposure to heterocyclic amines in the context of colon and bladder cancer has been well studied. In a prospective study, Chen *et al*. showed a trend of association of colon cancer as a function of amount of red meat consumption for rapid acetylators determined by NAT1 and NAT2 SNP variants [30]. In yet another prospective study with a female population, Chan *et al*. observed a high association between rapid acetylator phenotype as determined by

variants on the NAT2 gene and colon cancer, both as a function of meat consumption and smoking independently [31]. The association between colon cancer grew even stronger when considering those who both consumed higher amounts of meat and smoked [31]. On the other side of the coin, slow acetylation as determined by NAT2 SNP genotypes and exposure to aromatic amines—specifically arylamines found in cigarette smoke—have been associated with bladder cancer using pooled data [32, 33] in white populations. While the molecular mechanisms elucidating both slow and rapid acetylation in context of bladder and colorectal is not well understood, the general assumption has been that the carcinogenic activity is initiated by DNA adduction by these NAT-specific transformed products [34].

Genetic variations in GSTs are another large set of class II "antioxidant" enzymes implicated in adverse phenotypes, particularly in the context of the respiratory system and the inflammatory response. Specifically, an increase in water solubility is attained through conjugation of a glutathione group to an electrophilic portion—such as a reactive oxygen species—of a compound. Environmental exposures associated with these reactive species include those emerging from combustion and routed directly to the respiratory system, such as tobacco and diesel. For example, in a landmark (albeit with low sample size), randomized study investigating allergy, individuals with either the null genotype for GSTM1 (lacking complete activity) or the wild type for GSTM genes showed an "enhanced allergic response" to diesel pollutant fumes [35]. Further, these genotypes have been estimated to be highly prevalent, with frequencies greater than 40%, pointing to potential for high prevalence of enhanced allergic response under this mechanism according to the original investigators; however, others have argued recently for the larger role of GST genes in asthma [36]. Physiologically, the change in activity of these enzymes due to these variants leads to an accumulation of reactive species, initiating the inflammatory response, with damages due to the oxidative stress and inflammation.

The oxidative response of GSTM class of enzymes to prevalent exposures, such as smoking, has been studied at length in the context of cancers, particularly lung, prostate, and breast cancers. Specifically, in lung cancer, the presence of the null genotype leads to a moderate increase in risk for the disease in presence of tobacco smoke [37], due to smoke component benzo[*a*]pyrene, a highly carcinogenic and DNA disrupting PAH. As in the above example, individuals with the null genotype of GSTM1 cannot oxidize this compound efficiently, leading to—among other events—an increase in frequency of adduction events around hot spots in the cancer regulator and tumor suppressor p53 [38].

Other Examples of Processing Enzymes

Besides the class I and class II xenobiotic metabolism enzymes, there are other examples of gene products that harbor variants that affect their efficiency. Some of these are involved in processing of common food components, such as the alcohol dehydrogenase (ADH3) involved in the oxidation of ethanol and methylenetetrahydrofolate reductase (MTHFR) involved in the processing of the vitamin

folate toward building amino acid methionine. Yet another, the paraoxonase (PON1) is involved with the hydrolysis of organophosphate pesticides.

Analogous to our previous examples in which variants affected the rate of NAT or GSTM function, the γ-variants of the ADH3 gene encoding alcohol dehydrogenase affect the rate of ethanol oxidation. Those who are homozygous with the γ1 or γ2 allele are known as rapid or slow metabolizers of ethanol respectively. Consequently, the ADH3 genotype and high ethanol consumption have been observed to be associated with raised high-density lipoprotein cholesterol levels (HDL-C), which confers lowered risk to cardiovascular-related disease such as myocardial infarction [39]. For example, Hines *et al*. observed a sizable reduction of risk for myocardial infarction dependent jointly on moderate alcohol consumption (one glass per day) and the slow alcohol oxidation genotype [40]. It is unclear how the accrual of alcohol leads to increase of HDL-C. Some have shown that alcohol influences the transport rate of components of HDL-C, apolipoproteins apoI and apoII likely brought about by an increase in their production in the liver [41].

MTHFR is a gene that codes for the methylenetetrahydrofolate reductase enzyme, involved in the folate processing pathway; specifically, it converts a processed form of folate, 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate which is, in turn, processed by another reductase to convert the amino acid homocysteine to the amino acid methionine. Genetic variants of MTHFR have been at the crux of the study of many diseases, including colorectal cancer [42], coronary heart disease [43], and schizophrenia [44], in addition to congenital heart disease of offspring whose mothers are positive for a polymorphism of MTHFR [45]. One such nonsynonymous SNP variant results in a change of amino acid from alanine to valine (C677T; allele frequency highest among Hispanic and Caucasian ethnicities). The efficiency of the reductase activity is compromised for those who are homozygous for this variant and, as expected, there is a folate-dependent buildup of homocysteine [46]. From observational studies, it was noted that individuals with elevated homocysteine levels—mediated by MTFHR C677T genotype and folate levels—heightened risk for coronary artery disease [43]; nevertheless, in a deeper meta-analysis accounting for geography and ethnicity, a strong relation between the interaction and mitigation of coronary artery disease has been refuted [47], albeit the debate is ongoing [48, 49]. In contrast, with colorectal cancer, the C677T polymorphism and adequate folate levels proved to be protective. Furthermore, the protective effect of the genotype diminished for individuals with folate deficiency [42]. The investigators claim that this type of interaction may point to the role of methylation and DNA synthesis and repair in the etiology of colon cancer. From just one pair of an exposure and genetic variant, differential response may evidently affect risk for very different diseases.

The PON1 enzyme is named after paraoxon, a metabolite breakdown product of the insecticide parathion. PON1 functions to hydrolyze the oxidized organophosphates within the plasma, where it associated with HDL particles [50]. Examples of common organophosphate pesticides associated with PON1 processing include parathion and chlorpyrifos. The enzyme is also involved in directly processing nerve agents, such as sarin. As reviewed by Costa and colleagues, PON1 exhibited a “classic” bimodal pharmacokinetic activity distribution within a Caucasian population in which a one subset of individuals had drastically lower activity than another subset [51]. Several

polymorphisms of PON have been identified, and notably, a synonymous polymorphism resulting in a change in amino acid glutamine to arginine (Q192R)—a variant frequency estimated to be 25–65% depending on ethnicity [52]—is associated with the greatest difference in catalytic effect. However, the degree of this difference is of course dependent on the type of chemical substrate [51]. Due to its association in the serum with HDL, the Q192R variant of PON1 has been shown to play a marginal role in coronary artery disease (CAD) [53], but evidence of large effect has been scant [54]. Nevertheless, a joint role of the exogenous PON1 substrates and variants contributing to the etiology of a complex disease like CAD is a provocative possibility. In a recent study involving organophosphate applicators, another rarer coding polymorphism, leucine to methionine (L55M, 5–40% variant frequency depending on ethnicity), has been associated with Parkinson's disease [55]; however, this polymorphism is not related to change in catalytic activity but with actual plasma levels of the enzyme [51]. Interestingly, no significant findings have been found with other polymorphisms.

As expected, multiple interacting variants and exposures play a role in adverse phenotypes. For example, Polonikov *et al*., in a recent study of genetic risk for bronchial asthma in a Russian population, found that cigarette smoke played a role in asthma outcome only when conditioned on the absence of genotypes of two class I metabolic genes, CYP1A1 (462IV) and CYP1B1 (432LL) [56]. However, in addition to these conclusions regarding the gene–environment interaction of smoking and class I enzyme genotypes, the investigators found evidence for gene–gene interactions—the interactive effect of specific pair of genetic variants—in other notable xenobiotic metabolizing enzymes, such as PON2 and NAT2. As discussed above, these genes may also participate in gene–environment interactions, painting a complex etiological picture for asthma. Individually, while associations found in this study were of modest effect size, one is led to hypothesize that considering multiple exposures and genotypes in the aggregate could be more predictive of disease. Similarly, multiple exposures could also modify risk for disease. Extending on discussions above regarding NAT2 and colorectal cancers, one study using a population of Caucasians and Japanese immigrants to Hawaii found that individuals who were rapid acetylators (determined by either NAT2 or CYP1A2 genotype) and had a preference for both well-done meat and smoking resulted in an approximately ninefold increase of risk for colon cancer, compared to slow acetylators with no preference for well-done meat or smoking [57]. Simpler interactions between variant of NAT2 and CYP1A1 and either smoking or meat preference were inconclusive with regard to risk for cancer.

Variants Leading to a Change in Pharmacodynamic Response to Environmental Chemicals

The above discussion covered changes in metabolism, or chemical kinetics, due to genetic variation. While many results of the traditional investigations have illuminated the kinetic response, there are limited examples in which target host response, or

pharmacodynamics, is modulated by a genetic variant. One such example is the interaction between the variant of the protein that is involved with the immune response, HLA class II histocompatibility antigen (HLA-DPB1), and beryllium, resulting in greater risk for beryllium lung disease [58]. Chronic beryllium disease (CBD) is a chronic and sometimes lethal disorder that occurs in 1–10% of those exposed to certain forms of beryllium occupationally, even at very low levels. As a result of constant exposure, a massive immune response occurs in the lung, causing granulomas and scarring. According to Maier, a coding variant in a gene (67Glu, or change to glutamate) has been implicated in the immune response to beryllium exposure and is associated with much higher individual risk of the disease among individuals with this genotype. Hypothetically, this comes as a result of change in binding efficiency of the beryllium to HLA-DB1, leading to immune hypersensitivity [58].

Elucidating Interactions and Responses with Environmental Chemical Exposures on a Genome-Wide Scale

Availability of new genome-wide technologies and data sources has enabled novel and/or unbiased (i.e., nonhypothesis driven) methods to query for gene–environment interactions. We cover some of these methods, including the genome-wide association study (GWAS), systems genetics of gene–environment interaction, and informatics-based methods utilizing publicly available data to make predictions involving interaction of chemicals and genes.

GWAS have been proved to be a popular study design to comprehensively survey common DNA variants across the entire genome and their association with complex phenotypes such as disease [59–61]. In these studies, SNP "chips" are used to assay the genotypes of many individuals of common polymorphisms across the genome, and each locus is independently correlated with the phenotype of the individual (e.g., disease *vs.* nondisease, level of continuous clinical attribute, etc). Finally, after consideration of the fact that multiple hypotheses are in essence being tested simultaneously, the highest-ranking or most statistically significant loci are picked for further review, interpretation, and validation. As we would study any other phenotype, GWAS can be framed to scan for variants associated to response to chemicals. For example, investigators have initiated GWAS to scan for variants associated with the quantitative pharmacological response to drugs such as warfarin and antihypertensives [62]. Provided one can measure accurately phenotypic response to environmental chemicals and access an adequate number of individuals, GWAS can be applied to search for variants associated with response to environmental stimuli, such as number of cigarettes an individual smoker consumes per day [63–65]. In these large studies involving over 100,000 individuals and over 100 investigators from three consortia, the investigators were able to find genetic loci within genes that appear to mediate some aspects of smoking behavior, such as smoking initiation, intensity, and cessation ability. For example, the authors found high association around the cluster of genes called CHRNA3, CHRNA5,

and CHRNB4, which encode nicotinic acetylcholine receptors. When nicotine binds these receptors, it induces an increase of the neurotransmitter dopamine (among others), the "reward center" of the brain. The investigators also found variants within regions of genes such as BDNF and DBH, which they associated with smoking initiation and cessation behavior. Finally, the investigators found a "hot spot" of polymorphisms around a class I metabolic enzyme, CYP2A6. Analogous other class I enzymes discussed earlier, variants of CYP2A6 influence rate of catalysis of nicotine, individuals who are "rapid metabolizers" require more nicotine than "slow metabolizers" to achieve the same biologic effect. With GWAS, the investigators were able to systematically find variants that potentially describe molecular response to nicotine covering biological processes from metabolism to neurotransmission.

In the GWAS framework described above, we assume one can precisely observe the change in phenotype attributed solely to environmental exposure, such as the amount of cigarettes smoked. What happens if our phenotype of interest, such as disease, is complex a function of many processes other than exposure? To this end, some have proposed an analytic framework loosely termed "GEWIS," or gene–environment-wide interaction study [66], in which phenotype is modeled as a combination of both the environmental factor and each locus on the SNP array. Concretely, interaction between the chemical factor and genetic variant is analytically tested for each genetic locus. To our knowledge as of this writing, no GEWIS have been published; however, for a description of the methodology and constraints, one is encouraged to read Duncan Thomas' detailed review [67].

Romanoski and others have described an *in vitro* method to conduct a "systems genetics" analysis of gene–environment interactions and their effect on mechanisms of atherosclerosis [68]. In their study, they tried to study how the mechanism of vascular inflammation is dependent on both oxidized lipids and genetic variants. To conduct the study, the investigators developed aortic cell culture from 96 donors and treated them with a component of oxidized low-density lipoprotein, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphatidylcholine (Ox-PAPC), which induces a well-described inflammatory response. As their response phenotypes, they utilized genome-wide measurements of gene expression and conducted what is popularly known as an "expression quantitative trait loci" (eQTL) analysis. In eQTL analysis, genetic variants are comprehensively linked with genome-wide transcript levels, associating genetic variants with regulation of expression. The primary finding of this study included that approximately one-third of the highly regulated expression traits were significantly associated with interactions between genotype and exposure. The investigators uncovered complex "regulatory networks" dependent on the interaction and biologically validated the chain of regulatory events. For example, they found associations of interacting loci that were strongly related to with gene expression patterns "locally" and "distally." "Local" association denotes that the interacting locus is located physically close to the gene coding for expression and "distal" association denotes the opposite. Finally, a chain of regulatory events were inferred—and validated—between the distally and locally expressed genes. For example, they found an Ox-PAPC interacting locus strongly associated with expression of a gene that regulated the endoplasmic reticulum stress stimuli response.

Previous to their finding, it was known that Ox-PAPC induced these stress stimuli, but it was unknown how a genetic variant along with the exposure might lead to such a response. Specifically, they found the cell cycle regulator USP16 gene expression was modulated by the interacting locus locally. Local USP16 gene expression appeared to regulate the expression of six distal genes that were functionally related to endoplasmic reticulum stress stimuli, confirmed through a gene knockdown experiment. Notably, they observed a system of downstream molecular responses to interacting chemical exposures and variants that have eluded the traditional one-gene approach described in the first section above. However, there are some practical drawbacks to this approach, one of which includes that ethnicity was not taken into account; as we observed in the first section of this review, baseline ethnic diversity plays a large role in the association of variants to phenotype.

Audouze *et al*. [69] and Patel *et al*. [70] have proposed informatics-based approaches to predict environmental chemicals associated with complex disease using publicly available knowledge and data such as that from the Comparative Toxicogenomics Database (CTD) [71], the largest repository for gene expression data known as the Gene Expression Omnibus (GEO) [72], and protein–protein interaction data [73]. These methods contrast with those described above in that they are unbiased methods used for analytical prediction, testing many hypotheses simultaneously and leaving the best "predictions" for typical population-based studies. The CTD contains manually curated, qualitative knowledge of physical interactions between chemicals and genes based upon experiments on cell lines and model organisms [74]; interactions coded in the CTD include chemical–protein binding events, chemical-induced gene or protein expression changes, and chemical-DNA mutagenesis (among other interactions). Using the CTD, one of the authors (CJP) compiled 1,400 "gene-chemical signatures," sets of genes whose expression was associated with chemical exposure. We downloaded disease-associated gene expression data whose differential gene expression state could be attributed to the disease status of the samples and measured the correlation between each of the chemical-gene signatures and disease-associated gene expression patterns, hypothesizing that high correlation between a chemical-gene signature and disease expression activation state would suggest a potential environmental association. We found significant correlation between gene expression of key regulators such as BCL2, MAPK, and TNF induced by the chemotherapy doxorubicin and prostate cancer gene expression. As another example, we found significant correlation between expression signature of dimethylnitrosamine, a component of cigarette smoke, and lung cancer via genes such as growth factor TGFB1 and DNA synthesis-associated factor PCNA. The next step includes testing the combination of variants in these genes and chemicals in population-based studies.

In contrast to the above, Audouze *et al.* [69] used a "network pharmacology" approach to predict phenotypes that are associated with interacting chemicals and proteins. Here, the investigators utilized the CTD to extract chemical–protein binding events and inferred a protein–protein "association network" of those proteins that were connected by common chemicals. This novel association network was then compared to and validated by publicly available protein–protein interaction networks that had

been assembled using a priori published experimental data. Using computational network processing techniques, they were able to discriminate functional protein subnetworks into units called clusters. As a result of these procedures, the investigators had a set of interacting protein clusters and environmental chemicals associated with these clusters. Next, they computed the degree of association of each protein cluster to disease by mapping protein-disease information found in another publicly available database, GeneCards [75]. Finally, they used this cluster-disease-chemical map to evaluate chemicals potentially associated to disease. For example, they found as true positives a 128 protein cluster associated with breast cancer and chemicals such as polychlorinated biphenyls, bisphenol A, and genistein. The investigators also proposed utilizing the protein association network to predict novel targets of chemicals. Many environmental chemicals are known to be "promiscuous," i.e., they act on multiple proteins. For example, many chemicals that are known to bind estrogen receptor (ER) also bind to androgen receptor (AR). In addition, proteins that share a physical interaction with a chemical may also share interactions with chemicals sharing similar properties [76]. In order to identify novel targets for a particular chemical, the investigators used a method analogous to a "nearest neighbors" approach to make predictions about novel interactions using their protein association network. To illustrate, if chemicals A and B both bind proteins X and Y and a chemical C is also known to bind protein Y, their algorithm would predict C also binds X. In this way, they were able to predict novel chemical–protein binding events that did not exist in their database. One of these included the binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) to protein kinase C, PRKCE. Another strong finding included the prediction of binding between the highly prevalent plasticizer, di-(2-ethylhexyl) phthalate (DEHP) to γ-aminobutyric acid (GABA) neurotransmitter receptor, supported by recent evidence that DEHP could modulate functions of ion channels, such as GABA.

A major drawback of these bioinformatics methods is the lack of directionality of predicted phenotype associations: there is no way of discriminating between protective or harmful effects of the association as would be ascertained through epidemiological or toxicological studies. Nevertheless, these methods have proved to be a "scalable" means of creating data-driven hypotheses to validate using more conventional (toxicological or epidemiological) means.

New Paradigms

Perhaps ironically, the intense effort to explain interindividual differences in disease risks by GWAS described above has provided a major new rationale for focusing on the entire gamut of gene–environment interactions, while at the same time offering a paradigm for how to proceed. The rationale is straightforward: GWAS have thus far *failed* to explain but a small fraction of the variability in chronic disease risk predicted, based on twin studies, to be genetically mediated. Estimates for most chronic diseases, with the exception of macular degeneration, range between 1% and 10% of "genetic" variance explained by prevalent minor SNPs. While dozens of "risk conferring" and

"protective" SNPs have been confirmed, their collective explanatory power is very low. Among those still compelled by GWAS, two approaches are under active pursuit. First, larger and larger consortia are being assembled to improve the sensitivity and specificity of GWAS. There is also hope that with larger populations, some "high effect size, rare polymorphisms" might emerge (i.e., previously unidentified Mendelian traits). The number of new SNPs and refinements of point estimates from this effort has been impressive [77], but little new variance has succumbed to direct explanation. Indeed, increasing sample size might facilitate capturing additional variance but likely still fall far short of sibling relative risk estimates [78, 79]. Further, additional variance may be attributed by considering SNPs simultaneously, but variance attributable to specific genetic variants will remain undetected [80]. Second, the drive to reduce the cost of mass sequencing has accelerated. Many predict that substantial portions of the missing variance will be found to reside on non-SNP, high-predictive-value alleles such as the breast cancer gene (BRCA) for reproductive cancers. Yet there is increasing scientific acknowledgment, based on population observations studies showing that disease rates that vary enormously *within* genetically similar populations as a function of time, place, and social standing, that full sequencing will not provide the elusive explanation either [81]. Elucidation of gene–environment interactions more plausibly holds the key to understanding much of the "missing" genetic variation: i.e., common alleles whose clinical expression is triggered by common—but not ubiquitous—environmental stimuli, resulting in combination large effects sizes which could readily have been overlooked while examining either alone [82].

Unfortunately, formidable barriers limit our ability to proceed. Firstly, the "environment" is an extremely complex and dynamic soup of factors including not only the tens of thousands man-made and natural chemicals that have been the domain of toxicologists considered at the beginning of this chapter but also multiple prevalent physical factors (noise, electromagnetic radiations of all frequencies, heat, *etc*.), myriad infectious agents, including those residing in our gastrointestinal tract and mucosa, and, perhaps most vexing of all, "social" experiences, usually reduced to the single word "stress" in common parlance but likely a very complex set of exposures impacting divergent biologic pathways with multifold consequences. Unlike the genetic blueprint itself, characterization of these diverse elements cannot be reduced to analysis of a single biochemical medium such as DNA. It is the assessment of exposure to these diverse factors at the individual level in a consistent, affordable, reasonably valid, and high-throughput way for application to large populations whose clinical phenotypes are known that is now the paramount challenge.

Several promising approaches have recently emerged for quantifying the "environome," a concept coined 15 years ago by Anthony and colleagues [83].[1] As far back as the early 1980s, Pereira [86, 87] identified stable DNA-bound adducts of several

[1]Several other terms have been proposed for similar purposes. *Toxome* [84] would appear to suffer from being too narrow. *Exposome* [85] comes closer but may not be a useful way to think, for example, about the microbes living within us or our social interaction.

common environmental pollutants and proved them associated both to exposure (measured classically) and risk of cancers previously implicated by epidemiologic studies. Advances in technology have led to the search for further environmental "marks"—DNA methylation or the formation of adducts between environmental factors and genetic material—as means to quantify past exposure. While much of the research in epigenetics (see above) has focused on the heritable aspects of such changes, the technology has already received some impetus as a potential high-throughput way to characterize individual exposures to important hazards [14–16, 18, 19].

Early gene expression profiling, using Northern blots for single genes, was used primarily to study mechanisms of disease and prognosis. Now *expression* of large portions of the genome can also be studied in timed specimens to "fingerprint" exposures. Such has been the approach of Lamb and his colleagues who have profiled the collective changes induced by exposure to small molecules, dubbed "perturbagens," by studying *ex vivo* exposures of cell lines after exogenous dosing [88]. While efforts to connect these molecules to disease may be premature, the strategy represents a potential tool for characterizing yet another piece of the (unmeasured) human envirome. Gibson and his colleagues have employed an approach of this kind to elucidate characteristic "patterns"—presumable stable over time—of RNA expression, measurable on multigene arrays in genetically similar but socially distinct populations in remote areas of the world [89]. One intriguing aspect is that broad social/behavioral patterns were distinguished, auguring the possibility this may be one approach to finding biomarkers of "social environment," where existing measures—assessment of neural and hormonal responses to stress—remain fairly limited for the proposed large-population studies [90, 91]. One noteworthy advance in that regard has been a new focus "upstream" of the hormonal and inflammatory processes consequent to repetitive stress; Miller *et al*. have just published evidence that hypothalamic–pituitary–adrenocortical (HPA) axis dysregulation can be detected in differential prevalence of response elements in the promotor regions of sites such as glucocorticoid receptor [92]. Of particular interest is the observation that this measure could distinguish *historic* differences in stress exposures among adults whose current levels of stress were comparable. Overall, though, it remains premature to judge the potential for these techniques to be specific enough to classify *definable* exposures and quantitative enough to suit the needs of the proposed research paradigm.

Recently one of the authors (CJP) introduced "EWAS"—Environment-wide Association Studies—which exploited the research strategy of "GWAS" by testing the unbiased associations between blood and urine measures of chemicals and disease in the last four surveys of the National Health and Nutrition Examination Survey (NHANES) [93]. Far above the level that "false discovery" is a likely explanation, five different compounds appeared in multiple surveys to be strongly associated with diabetes mellitus, the first disease tested. Promising as the strategy may be, it will not by itself provide the path to the full human "envirome." For one

thing, only a modest number of chemical contaminants remain in the environment or human body in measurable form; even if the cost could be reduced, the extent of application of direct *in vivo* measurement of hazards will be limited. More promising may be new technologies to assess microbial environment, simultaneously revealing the presence or absence of tens of thousands of species [94, 95]. The use of these tools to characterize intestinal and other mucosal flora—potentially an environment of some considerable interest in gene–environment interaction—is noteworthy and promising as an approach to elucidation of the human envirome.

Promising as they are, EWAS, like the other approaches described above, highlight a generic problem which will complicate all gene–environment interaction studies: the problem of distinguishing *causality* from the two other explanations for statistical association: *reverse causality*—disease leading to accumulation of an exposure—or the possibility that both disease and exposure are related to a third *unmeasured* factor, i.e., *confounding*, harder and harder to assess as the number of factors—the breadth of the envirome, so to speak—expands [96]. A case in point is the reported association from NHANES between diabetes and bisphenol A [97], likely both related to an unhealthful diet since the major source of the toxin in adults is canned foodstuffs. This explanation is suggested by a striking inverse relationship in the same dataset between toxin levels in urine and β-carotene in blood. Such methodological difficulties far less complicate interpretation of GWAS once race/ethnicity is balanced between cases and controls since the genetic factors always precede clinical phenotype in time and do not change, at least not much, epigenetic findings notwithstanding.

In fact, knowledge that genetic structure precedes other causes may be exploited to help manage the issues of reverse causality and confounding in gene–environment studies through a new technique dubbed "Mendelian randomization." The idea is to analyze associations between an environmental factor and disease exploiting, as an "instrumental variable," a gene believed a priori to act via an exposure related mechanism. For example, where a minor allele is known to delay (or enhance) the metabolism of a toxin of interest, or upregulate receptor responses, the random assignment of the these genes allows a virtual randomized controlled trial to evaluate toxic effect among those with otherwise comparable levels of exposure, assuming this is the major pathway by which the gene influences disease risk. This analytic technique may also be useful for ranking the possibility of gene–environment interactions for alleles conferring disease risk but for which the mechanism of effect is entirely unknown: comparison of the gene–-disease associations before *vs*. after adjusting for exposure may provide a hint as to whether the environmental factor is or is not related to the gene's mode of action, depending to what degree the adjustment extinguishes the excess risk.

This approach and other forms of Bayesian analysis to manage confounding and reverse causality, however, must remain backseat considerations until the biggest gap in this ambitious plan is filled by a practicable, broad-based, consistent, valid, high-throughput set of tools to measure the envirome on large populations.

Conclusion and Discussion

In the above, we have described and discussed examples of paradigms to examine, or search for, the joint effects of genetic variants and environmental exposures. Since the inception of gene–environment interaction studies, most investigations have been concerned about finding the interaction effect of single genetic variants and exposures in the biological context of chemical metabolism and kinetics. Since then, the search for variants beyond metabolism genes has expanded with genome-wide measurement modalities and "hypothesis-generating" bioinformatics and computational methods. Finally, we argue that in the pursuit to find missing heritability for disease, our representation of chemical factors must be expanded to the breadth of an envirome—a concept analogous to genome—that enable comprehensive queries over both the space of genes and the environment.

Many challenges remain for the field. Compared to the latest genome-wide disease association studies, validated studies considering the joint effect of genetic variants and environmental chemical factors have been lacking [66]. This is not surprising: analytically and logistically, studying interactions on an epidemiological level for non-Mendelian complex diseases will be resource and sample intensive [67]. Second, there has been lack of bidirectional data and knowledge flow between the newest high-throughput toxicology screens and epidemiological, human population-based studies; mapping complex toxicological response to complex human disease has only just begun. Last, the domain of possible known environmental factors that might play a role in gene–environment interactions may outnumber the number of genes in the genome. Therefore, there is need for efforts to define the domain of the envirome much as we did when we sequenced the genome. Novel methods, study designs, and datasets need to be developed to enable a comprehensive survey of these factors simultaneously, all the while tackling the major issues of current epidemiology and toxicology such as confounding, replication, and translation. To gain public health relevance and to step closer to the deciphering etiology of complex diseases and phenotypes, future studies will have to integrate data over both the genome and the envirome.

Acknowledgments C.J.P. was funded by the National Library of Medicine (T15 LM 007033).

References

1. NICHD (2010) Phenylketonuria. National Institute of Child Health and Human Development, National Institutes of Health (NIH), Bethesda, MD. Online available at http://www.nichd.nih.gov/health/topics/phenylketonuria.cfm
2. Garrod A (1902) Alkaptonuria. Lancet: 653–656
3. Garrod A (1931) The inborn factors in disease: an essay. Clarendon, Oxford, UK
4. Motulsky AG (1957) Drug reactions, enzymes, and biochemical genetics. J Am Med Assoc 165:835–837
5. International HapMap Consortium (2005) A haplotype map of the human genome. Nature 437:1299–1320

6. Kidd JM, Cooper GM, Donahue WF, Hayden HS, Sampas N, Graves T, Hansen N, Teague B, Alkan C, Antonacci F *et al* (2008) Mapping and sequencing of structural variation from eight human genomes. Nature 453:56–64
7. Ellegren H (2004) Microsatellites: simple sequences with complex evolution. Nat Rev Genet 5:435–445
8. NCBI (2010) dbSNP. Short Genetic Variations. National Center for Biotechnology Information. Online available at http://www.ncbi.nlm.nih.gov/projects/SNP/
9. Tost J (2008) Epigenetics. Caister Academic, Norfolk
10. Reik W, Dean W, Walter J (2001) Epigenetic reprogramming in mammalian development. Science 293:1089–1093
11. Sasaki H, Matsui Y (2008) Epigenetic events in mammalian germ-cell development: reprogramming and beyond. Nat Rev Genet 9:129–140
12. Whitelaw NC, Whitelaw E (2008) Transgenerational epigenetic inheritance in health and disease. Curr Opin Genet Dev 18:273–279
13. Bollati V, Baccarelli A (2010) Environmental epigenetics. Heredity 105:105–112
14. Esteller M (2008) Epigenetics in cancer. New Engl J Med 358:1148–1159
15. Baccarelli A, Wright RO, Bollati V, Tarantini L, Litonjua AA, Suh HH, Zanobetti A, Sparrow D, Vokonas PS, Schwartz J (2009) Rapid DNA methylation changes after exposure to traffic particles. Am J Respir Crit Care Med 179:572–578
16. Rusiecki JA, Baccarelli A, Bollati V, Tarantini L, Moore LE, Bonefeld-Jorgensen EC (2008) Global DNA hypomethylation is associated with high serum-persistent organic pollutants in Greenlandic Inuit. Environ Health Perspect 116:1547–1552
17. Tarantini L, Bonzini M, Apostoli P, Pegoraro V, Bollati V, Marinelli B, Cantone L, Rizzo G, Hou L, Schwartz J, Bertazzi PA, Baccarelli A (2008) Effects of particulate matter on genomic DNA methylation content and *iNOS* promoter methylation. Environ Health Perspect 117: 217–222
18. Wright RO, Schwartz J, Wright RJ, Bollati V, Tarantini L, Park SK, Hu H, Sparrow D, Vokonas P, Baccarelli A (2010) Biomarkers of lead exposure and DNA methylation within retrotransposons. Environ Health Perspect 118:790–795
19. Baccarelli A, Bollati V (2009) Epigenetics and environmental chemicals. Curr Opin Pediatr 21:243–251
20. Khoury MJ, Beaty TH, Cohen B (1993) Fundamentals of genetic epidemiology. Oxford University Press, New York, NY
21. Khoury MJ, Adams MJ Jr, Flanders WD (1988) An epidemiologic approach to ecogenetics. Am J Hum Genet 42:89–95
22. Siemiatycki J, Thomas DC (1981) Biological models and statistical interactions: an example from multistage carcinogenesis. Int J Epidemiol 10:383–387
23. Rothman K, Greenland S, Lash T (2008) Modern epidemiology, 3rd edn. Lippincott Williams & Wilkins, Philadelphia, PA
24. Phornphutkul C, Introne WJ, Perry MB, Bernardini I, Murphey MD, Fitzpatrick DL, Anderson PD, Huizing M, Anikster Y, Gerber LH, Gahl WA (2002) Natural history of alkaptonuria. New Engl J Med 347:2111–2121
25. Nelson D (2009) The cytochrome P450 homepage. Hum Genomics 4:59–65
26. Nebert DW, Dalton TP, Okey AB, Gonzalez FJ (2004) Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. J Biol Chem 279:23847–23850
27. Badawi AF, Cavalieri EL, Rogan EG (2001) Role of human cytochrome P450 1A1, 1A2, 1B1, and 3A4 in the 2-, 4-, and 16α-hydroxylation of 17β-estradiol. Metabolism 50: 1001–1003
28. Kellermann G, Shaw CR, Luyten-Kellerman M (1973) Aryl hydrocarbon hydroxylase inducibility and bronchogenic carcinoma. N Engl J Med 289:934–937
29. Crofts F, Taioli E, Trachman J, Cosma GN, Currie D, Toniolo P, Garte SJ (1994) Functional significance of different human CYP1A1 genotypes. Carcinogenesis 15:2961–2963

30. Chen J, Stampfer MJ, Hough HL, Garcia-Closas M, Willett WC, Hennekens CH, Kelsey KT, Hunter DJ (1998) A prospective study of *N*-acetyltransferase genotype, red meat intake, and risk of colorectal cancer. Cancer Res 58:3307–3311
31. Chan AT, Tranah GJ, Giovannucci EL, Willett WC, Hunter DJ, Fuchs CS (2005) Prospective study of *N*-acetyltransferase-2 genotypes, meat intake, smoking and risk of colorectal cancer. Int J Cancer 115:648–652
32. Garcia-Closas M, Malats N, Silverman D, Dosemeci M, Kogevinas M, Hein DW, Tardon A, Serra C, Carrato A, Garcia-Closas R, Lloreta J, Castano-Vinyals G, Yeager M, Welch R, Chanock S, Chatterjee N, Wacholder S, Samanic C, Tora M, Fernandez F, Real FX, Rothman N (2005) NAT2 slow acetylation, GSTM1 null genotype, and risk of bladder cancer: results from the Spanish Bladder Cancer Study and meta-analyses. Lancet 366:649–659
33. Vineis P, Marinelli D, Autrup H, Brockmoller J, Cascorbi I, Daly AK, Golka K, Okkels H, Risch A, Rothman N, Sim E, Taioli E (2001) Current smoking, occupation, *N*-acetyltransferase-2 and bladder cancer: a pooled analysis of genotype-based studies. Cancer Epidemiol Biomarkers Prev 10:1249–1252
34. Hein DW, Doll MA, Fretland AJ, Leff MA, Webb SJ, Xiao GH, Devanaboyina US, Nangju NA, Feng Y (2000) Molecular genetics and epidemiology of the NAT1 and NAT2 acetylation polymorphisms. Cancer Epidemiol Biomarkers Prev 9:29–42
35. Gilliland FD, Li YF, Saxon A, Diaz-Sanchez D (2004) Effect of glutathione-*S*-transferase M1 and P1 genotypes on xenobiotic enhancement of allergic responses: randomised, placebo-controlled crossover study. Lancet 363:119–125
36. Minelli C, Granell R, Newson R, Rose-Zerilli MJ, Torrent M, Ring SM, Holloway JW, Shaheen SO, Henderson JA (2010) Glutathione-*S*-transferase genes and asthma phenotypes: a human genome epidemiology (HuGE) systematic review and meta-analysis including unpublished data. Int J Epidemiol 39:539–562
37. Benhamou S, Lee WJ, Alexandrie AK, Boffetta P, Bouchardy C, Butkiewicz D, Brockmoller J, Clapper ML, Daly A, Dolzan V *et al* (2002) Meta- and pooled analyses of the effects of glutathione *S*-transferase M1 polymorphisms and smoking on lung cancer risk. Carcinogenesis 23:1343–1350
38. Denissenko MF, Pao A, Tang M, Pfeifer GP (1996) Preferential formation of benzo[*a*]pyrene adducts at lung cancer mutational hotspots in P53. Science 274:430–432
39. Talmud PJ (2007) Gene-environment interaction and its impact on coronary heart disease risk. Nutr Metab Cardiovasc Dis 17:148–152
40. Hines LM, Stampfer MJ, Ma J, Gaziano JM, Ridker PM, Hankinson SE, Sacks F, Rimm EB, Hunter DJ (2001) Genetic variation in alcohol dehydrogenase and the beneficial effect of moderate alcohol consumption on myocardial infarction. N Engl J Med 344: 549–555
41. De Oliveira e Silva ER, Foster D, McGee Harper M, Seidman CE, Smith JD, Breslow JL, Brinton EA (2000) Alcohol consumption raises HDL cholesterol levels by increasing the transport rate of apolipoproteins A-I and A-II. Circulation 102:2347–2352
42. Chen J, Giovannucci EL, Hunter DJ (1999) MTHFR polymorphism, methyl-replete diets and the risk of colorectal carcinoma and adenoma among U.S. men and women: an example of gene-environment interactions in colorectal tumorigenesis. J Nutr 129:560S–564S
43. Klerk M, Verhoef P, Clarke R, Blom HJ, Kok FJ, Schouten EG (2002) MTHFR 677C→T polymorphism and risk of coronary heart disease: a meta-analysis. J Am Med Assoc 288: 2023–2031
44. Roffman JL, Weiss AP, Deckersbach T, Freudenreich O, Henderson DC, Purcell S, Wong DH, Halsted CH, Goff DC (2007) Effects of the methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism on executive function in schizophrenia. Schizophr Res 92: 181–188
45. van Beynum IM, Kapusta L, den Heijer M, Vermeulen SH, Kouwenberg M, Daniels O, Blom HJ (2006) Maternal MTHFR 677C>T is a risk factor for congenital heart defects: effect modification by periconceptional folate supplementation. Eur Heart J 27:981–987

46. Jacques PF, Bostom AG, Williams RR, Ellison RC, Eckfeldt JH, Rosenberg IH, Selhub J, Rozen R (1996) Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. Circulation 93:7–9
47. Lewis SJ, Ebrahim S, Davey Smith G (2005) Meta-analysis of MTHFR 677C→T polymorphism and coronary heart disease: does totality of evidence support causal role for homocysteine and preventive potential of folate? Br Med J 331:1053
48. Lonn E (2008) Homocysteine-lowering B vitamin therapy in cardiovascular prevention–Wrong again? J Am Med Assoc 299:2086–2087
49. Wald DS, Wald NJ, Morris JK, Law M (2006) Folic acid, homocysteine, and cardiovascular disease: judging causality in the face of inconclusive trial evidence. Br Med J 333: 1114–1117
50. Gaidukov LA, Aharoni A, Khersonsky O, Tawfik DS (2008) Serum paraoxonase PON1 and its interactions with HDL. FASEB J 22(Suppl): 811.1
51. Costa LG, Cole TB, Furlong CE (2003) Polymorphisms of paraoxonase (PON1) and their significance in clinical toxicology of organophosphates. J Toxicol Clin Toxicol 41:37–45
52. Ginsberg G, Neafsey P, Hattis D, Guyton KZ, Johns DO, Sonawane B (2009) Genetic polymorphism in paraoxonase 1 (PON1): population distribution of PON1 activity. J Toxicol Environ Health B Crit Rev 12:473–507
53. Sanghera DK, Aston CE, Saha N, Kamboh MI (1998) DNA polymorphisms in two paraoxonase genes (PON1 and PON2) are associated with the risk of coronary heart disease. Am J Hum Genet 62:36–44
54. Wheeler JG, Keavney BD, Watkins H, Collins R, Danesh J (2004) Four paraoxonase gene polymorphisms in 11212 cases of coronary heart disease and 12786 controls: meta-analysis of 43 studies. Lancet 363:689–695
55. Manthripragada AD, Costello S, Cockburn MG, Bronstein JM, Ritz B (2010) Paraoxonase 1, agricultural organophosphate exposure, and Parkinson disease. Epidemiology 21:87–94
56. Polonikov AV, Ivanov VP, Solodilova MA (2009) Genetic variation of genes for xenobiotic-metabolizing enzymes and risk of bronchial asthma: the importance of gene-gene and gene-environment interactions for disease susceptibility. J Hum Genet 54:440–449
57. Le Marchand L, Hankin JH, Wilkens LR, Pierce LM, Franke A, Kolonel LN, Seifried A, Custer LJ, Chang W, Lum-Jones A, Donlon T (2001) Combined effects of well-done red meat, smoking, and rapid *N*-acetyltransferase 2 and CYP1A2 phenotypes in increasing colorectal cancer risk. Cancer Epidemiol Biomarkers Prev 10:1259–1266
58. Maier LA (2002) Genetic and exposure risks for chronic beryllium disease. Clin Chest Med 23:827–839
59. Hirschhorn JN, Daly MJ (2005) Genome-wide association studies for common diseases and complex traits. Nat Rev Genet 6:95–108
60. McCarthy MI, Abecasis GR, Cardon LR, Goldstein DB, Little J, Ioannidis JPA, Hirschhorn JN (2008) Genome-wide association studies for complex traits: consensus, uncertainty and challenges. Nat Rev Genet 9:356–369
61. Pearson TA, Manolio TA (2008) How to interpret a genome-wide association study. J Am Med Assoc 299:1335–1344
62. Crowley JJ, Sullivan PF, McLeod HL (2009) Pharmacogenomic genome-wide association studies: lessons learned thus far. Pharmacogenomics 10:161–163
63. Liu JZ, Tozzi F, Waterworth DM, Pillai SG, Muglia P, Middleton L, Berrettini W, Knouff CW, Yuan X, Waeber G *et al* (2010) Meta-analysis and imputation refines the association of 15q25 with smoking quantity. Nat Genet 42:436–440
64. The Tobacco and Genetics Consortium (2010) Genome-wide meta-analyses identify multiple loci associated with smoking behavior. Nat Genet 42:441–447
65. Thorgeirsson TE, Gudbjartsson DF, Surakka I, Vink JM, Amin N, Geller F, Sulem P, Rafnar T, Esko T, Walter S *et al* (2010) Sequence variants at CHRNB3-CHRNA6 and CYP2A6 affect smoking behavior. Nat Genet 42:448–453

66. Khoury MJ, Wacholder S (2009) Invited commentary: from genome-wide association studies to gene-environment-wide interaction studies—challenges and opportunities. Am J Epidemiol 169:227–230, discussion 234–225
67. Thomas D (2010) Gene-environment-wide association studies: emerging approaches. Nat Rev Genet 11:259–272
68. Romanoski CE, Lee S, Kim MJ, Ingram-Drake L, Plaisier CL, Yordanova R, Tilford C, Guan B, He A, Gargalovic PS, Kirchgessner TG, Berliner JA, Lusis AJ (2010) Systems genetics analysis of gene-by-environment interactions in human cells. Am J Hum Genet 86:399–410
69. Audouze K, Juncker AS, Roque FJ, Krysiak-Baltyn K, Weinhold N, Taboureau O, Jensen TS, Brunak S (2010) Deciphering diseases and biological targets for environmental chemicals using toxicogenomics networks. PLoS Comput Biol 6:e1000788
70. Patel C, Butte A (2010) Predicting environmental chemical factors associated with disease-related gene expression data. BMC Med Genomics 3:17
71. Gohlke JM, Thomas R, Zhang Y, Rosenstein MC, Davis AP, Murphy C, Becker KG, Mattingly CJ, Portier CJ (2009) Genetic and environmental pathways to complex diseases. BMC Syst Biol 3:46
72. Barrett T, Troup DB, Wilhite SE, Ledoux P, Rudnev D, Evangelista C, Kim IF, Soboleva A, Tomashevsky M, Edgar R (2007) NCBI GEO: mining tens of millions of expression profiles—database and tools update. Nucleic Acids Res 35:D760–765
73. Lage K, Karlberg EO, Storling ZM, Olason PI, Pedersen AG, Rigina O, Hinsby AM, Tumer Z, Pociot F, Tommerup N, Moreau Y, Brunak S (2007) A human phenome-interactome network of protein complexes implicated in genetic disorders. Nat Biotechnol 25:309–316
74. Davis AP, Murphy CG, Saraceni-Richards CA, Rosenstein MC, Wiegers TC, Mattingly CJ (2009) Comparative Toxicogenomics Database: a knowledgebase and discovery tool for chemical-gene-disease networks. Nucleic Acids Res 37:D786–792
75. Rebhan M, Chalifa-Caspi V, Prilusky J, Lancet D (1997) GeneCards: integrating information about genes, proteins and diseases. Trends Genet 13:163
76. Keiser MJ, Roth BL, Armbruster BN, Ernsberger P, Irwin JJ, Shoichet BK (2007) Relating protein pharmacology by ligand chemistry. Nat Biotechnol 25:197–206
77. Tanya MT, Kiran M, Albert VS, Andrew CE, Ioannis MS, Masahiro K, James PP, Ripatti S, Daniel IC, Cristen JW *et al* (2010) Biological, clinical and population relevance of 95 loci for blood lipids. Nature 466:707
78. Park JH, Wacholder S, Gail MH, Peters U, Jacobs KB, Chanock SJ, Chatterjee N (2010) Estimation of effect size distribution from genome-wide association studies and implications for future discoveries. Nat Genet 42:570–575
79. Goldstein DB (2009) Common genetic variation and human traits. N Engl J Med 360: 1696–1698
80. Yang J, Benyamin B, McEvoy BP, Gordon S, Henders AK, Nyholt DR, Madden PA, Heath AC, Martin NG, Montgomery GW, Goddard ME, Visscher PM (2010) Common SNPs explain a large proportion of the heritability for human height. Nat Genet 42:565–569
81. Marmot MG, Smith GD, Stansfeld S, Patel C, North F, Head J, White I, Brunner E, Feeney A (1991) Health inequalities among British civil servants: the Whitehall II study. Lancet 337: 1387–1393
82. Schwartz D, Collins F (2007) Medicine. Environmental biology and human disease. Science 316:695–696
83. Anthony JC, Eaton WW, Henderson AS (1995) Looking to the future in psychiatric epidemiology. Epidemiol Rev 17:240–242
84. Human Toxome Project (2010) Environmental Working Group, Commonweal EWG. Online available at http://www.ewg.org/sites/humantoxome/
85. Wild CP (2005) Complementing the genome with an "exposome": the outstanding challenge of environmental exposure measurement in molecular epidemiology. Cancer Epidemiol Biomarkers Prev 14:1847–1850

86. Pereira MA, Burns FJ, Albert RE (1979) Dose response for benzo[*a*]pyrene adducts in mouse epidermal DNA. Cancer Res 39:2556–2559
87. Pereira MA, Lin LH, Chang LW (1981) Dose-dependency of 2-acetylaminofluorene binding to liver DNA and hemoglobin in mice and rats. Toxicol Appl Pharmacol 60:472–478
88. Lamb J, Crawford ED, Peck D, Modell JW, Blat IC, Wrobel MJ, Lerner J, Brunet JP, Subramanian A, Ross KN, Reich N, Hieronymus H, Wei G, Armstrong SA, Haggarty SJ, Clemons PA, Wei R, Carr SA, Lander ES, Golub TR (2006) The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease. Science 313: 1929–1935
89. Idaghdour Y, Czika W, Shianna KV, Lee SH, Visscher PM, Martin HC, Miclaus K, Jadallah SJ, Goldstein DB, Wolfinger RD, Gibson G (2010) Geographical genomics of human leukocyte gene expression variation in southern Morocco. Nat Genet 42:62–67
90. McEwen BS (1998) Stress, adaptation, and disease: allostasis and allostatic load. Ann NY Acad Sci 840:33–44
91. Seeman TE, McEwen BS, Rowe JW, Singer BH (2001) Allostatic load as a marker of cumulative biological risk: MacArthur studies of successful aging. Proc Natl Acad Sci USA 98:4770–4775
92. Miller GE, Chen E, Fok AK, Walker H, Lim A, Nicholls EF, Cole S, Kobor MS (2009) Low early-life social class leaves a biological residue manifested by decreased glucocorticoid and increased proinflammatory signaling. Proc Natl Acad Sci USA 106:14716–14721
93. Patel C, Bhattacharya J, Butte A (2010) An environment-wide association study (EWAS) to type 2 diabetes. PLoS One 5:e10746
94. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA (2005) Diversity of the human intestinal microbial flora. Science 308:1635–1638
95. Gill SR, Pop M, DeBoy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, Gordon JI, Relman DA, Fraser-Liggett CM, Nelson KE (2006) Metagenomic analysis of the human distal gut microbiome. Science 312:1355–1359
96. Ioannidis J, Loy EY, Poulton R, Chia KS (2009) Researching genetic *versus* nongenetic determinants of disease: a comparison and proposed unification. Sci Transl Med 1:8
97. Lang IA, Galloway TS, Scarlett A, Henley WE, Depledge M, Wallace RB, Melzer D (2008) Association of urinary bisphenol A concentration with medical disorders and laboratory abnormalities in adults. J Am Med Assoc 300:1303–1310

Biomarkers in Toxicology and Risk Assessment

Bruce A. Fowler

Abstract Over the last 30 years, the field of biomarkers has greatly expanded as early and specific endpoints for monitoring cellular responses to various disease states and exposures to drugs and chemical agents. They have enjoyed some success as predictors of health outcomes for a number of clinical diseases, but the application to chemical exposure risk assessments has been more limited. Biomarkers may be classified into categories of markers of exposure, effect, and susceptibility. Currently, "omics" biomarkers (i.e., genomic, proteomic, and metabolomic/metabonomic) are the major classes of biomarkers under development. These markers represent a continuum of cellular responses to drug or chemical exposures and provide linkages to mechanisms of cell injury/cell death or carcinogenic transformation. On the other hand, translation and application of these biomarkers for risk assessment has been limited due to validation and interpretation issues that need to be addressed in order for these potentially extremely valuable endpoints to reach their full potential as predictive tools for public health. This short chapter will briefly review these three "omics" biomarker classes and examine some validation/translation aspects needed in order for them to reach their full potential and acceptance as valuable tools for application to risk assessment.

Keywords Bioinformatics · Computational modeling · Genomic biomarkers · Metabolomic biomarkers · Mixtures · Nanomaterials · Proteomic biomarkers · Validation

Bruce A. Fowler (✉)
Division of Toxicology and Environmental Medicine, Agency for Toxic Substances and Disease Registry (ATSDR), Atlanta, GA 30333, USA
ICF International, Fairfax, VA, USA
e-mail: bfowler@icfi.com; drtox@earthlink.net

A. Luch (ed.), *Molecular, Clinical and Environmental Toxicology*,
Experientia Supplementum 101, DOI 10.1007/978-3-7643-8340-4_16,

Introduction

The term biomarker has evolved from its earliest definition as a "preclinical biological indicator" [1–5] to include several classes of biomarkers that play an ever-increasing role in mode of action-based risk assessment. In general, biomarkers may be classified into biomarkers of exposure, biomarkers of effect, and biomarkers of susceptibility [6–8]. Biomarkers represent a variety of measureable biological or molecular endpoints which are responsive to cellular exposures to chemicals, fibers such as asbestos [9], drugs, or disease processes [10, 11] including cancer [12]. The current classes of biomarkers of greatest scientific interest are globally referred to as "omics" biomarkers. These "omics" biomarkers include genomic (genotyping, gene expression, and epigenomic), proteomic, and metabolomic/metabonomic-based biomarkers [13] which will be discussed in greater detail below. Regardless of the particular biomarker endpoint evaluated, the overarching concept is that one can use basic scientific knowledge about how toxic chemicals interact with the essential cellular machinery to develop tests that objectively assess early cellular responses to chemical exposures and provide a scientific linkage between chemical exposures, early cellular effects, and a degree of prediction on likely long-term health consequences for risk assessment purposes. As noted below, a major challenge to the field of biomarker development is the need for rigorous validation endpoints so that the measured biomarker responses may be correctly interpreted and linked to mechanisms of chemical-induced cell injury/cell death. There have been a number of review articles [13–16] and conferences focused on biomarkers in recent years [17, 18] which have summarized these issues and suggested research needs.

It should also be noted that there are a number of biological factors such as antioxidants (e.g., glutathione), metal-binding proteins (e.g., metallothionein [19, 20], lead-binding proteins [21–24]), lead inclusion bodies [25], excision and repair enzymes, metabolizing enzyme systems, and nutritional, gender, age, genetic inheritance, and dose/duration of exposure factors which may influence biomarker responses. These factors are important for risk assessments since they help to define subpopulations at special risk but also point out the need for combining data from multiple biomarkers to develop a more complete picture of ongoing processes to better inform risk assessments [14, 15].

The following discussion briefly summarizes recent progress in the fields of genomic, proteomic, and metabolomic/metabonomic biomarkers and attempts to highlight how these major classes of biomarkers have been utilized to better understand mechanisms of toxicity and the extent to which they have been translated into mode of action-based risk assessments. It is important to note that while these "omics" classes will be covered on an individual basis, they are actually interrelated as components of integrated cellular biological systems. Hence, it is likely that responses in one of these classes will also be reflected in the other two classes. Ideally, it would be most informative to measure responses in all three classes at the same time and utilize the also evolving tools of computational modeling to provide integrative interpretational insights.

Genomic Biomarkers

Genomic biomarkers have been in use for the past 20 years and basically provide measures of cellular responses to chemical or drug exposures at the level of the gene with regard to modifications of specific genotypes (e.g., δ-aminolevulinic acid dehydratase, ALAD) up- or downregulation of specific genes (e.g., metallothionein, stress proteins) or epigenetic modifications such as altered methylation of DNA bases.

A recent useful example of where genotyping has been utilized for risk assessment are studies which examined the relationship between ALAD polymorphisms (ALAD 1 and ALAD 2) [26] and risk of lead-induced hypertension as reported in the NHANES database. These studies showed that white male ALAD 2 carriers had a greater risk of lead-induced hypertension than ALAD 1 carriers with greater effects at the highest blood lead quartile—in other words, with evidence of a dose effect. The value of these data for risk assessment is clear given the public health significance of hypertension and that these findings are reflective of the general US population.

Measurement of altered gene expression patterns is commonly accomplished via hybridization of messenger RNAs isolated from cells of interest with complementary DNA for those genes affixed to chips and use of fluorescent readouts. The technique is now rapid and relatively inexpensive and provides direct information on alterations in specific gene expression patterns from chemical or drug exposures. The limitations of the approach are that it will not pick up those genes not present on the chip and there is a well-known issue regarding genes showing increased expression on the chip but the products of those genes not actually being expressed as proteins. This possible disconnect may complicate interpretation of findings unless confirmatory data from proteomic studies are also available. This approach has been applied to human populations exposed to cadmium and expression of metallothionein [27]. On the other hand, genomic expression data may provide valuable insights as susceptibility biomarkers for genetic polymorphisms (e.g., ALAD; glutathione *S*-transferases, GSTs; cytochrome P450-dependent monooxygenases, CYPs) that are known to influence susceptibility to toxicities from both inorganic and organic toxic agents. This is important in helping to define sensitive subpopulations for risk assessment purposes. Epigenomic approaches [28] provide intriguing possibilities into how altered regulation of DNA activity may influence susceptibility and risk for toxicity and clinical diseases including cancer.

Mechanistic Linkages

The linkages between genomic biomarkers and mechanisms of chemical-induced toxicity or cancer rest with an appreciation of whether major regulatory genes such a p53 or proto-oncogenes and their attendant pathways are being up- or downregulated in response to a given chemical exposure. This is useful information for developing research protocols to further investigate health outcomes if these responses persist

following continued exposures. To this end, the application of computational models for pathway and network analyses, as discussed below, may be extremely valuable for delineating functional interactions between genes responding directly to chemical exposures and major regulatory genes linked to them either directly or indirectly.

Risk Assessment Linkages

The risk assessment linkages for the genomic biomarkers are a bit more tenuous as predictive markers of effects due to issues related to the persistence of responses and whether the activation of specific genes actually results in the production of gene products that are the effector molecules which result in altered cell biology leading to cell injury/cell death or cancer. The real value of genomic approaches for risk assessment most likely resides in evaluation of the roles of genomic polymorphisms in determining sensitive subpopulations at special risk and, in combination with computational modeling, helping to delineate gene–gene interactions via pathway and network analyses [29, 30]. This information is of great potential value in estimating risks for both cellular toxicity and cancer outcomes.

Proteomic Biomarkers

Proteomic biomarkers involve the assessment of changes in protein expression patterns via 2D gel electrophoresis or mass spectrometry and have the advantage of visualizing changes in the patterns of actual gene products in both cells of interest and accessible body fluids such as blood or urine [31]. This class of "omics" biomarker has been applied to both cancer [10, 32–35] and noncancer [36–38] endpoints for both monitoring existing disease and predicting outcomes. The value of such data is in providing direct mechanistic insights into both cellular responses in target cell populations, susceptibility issues based upon the expression of protective proteins, such as stress proteins or metallothionein, and measures of target organ toxicity such as urinary protein excretion patterns. In addition, it must be noted that expressed proteins represent the functional aspects of gene up- and downregulation in response to chemical exposures. The pattern of these changes and linkage to chemical-specific proteinuria patterns have been shown [17, 18, 38] to be reflective of target cell population responses to toxic chemicals on an individual and mixture basis and also reflective of dose and duration of exposure in experimental systems. Future progress in this area will be due in part to the development of improved bioinformatics and analysis software [38]. From the perspective of linking chemical exposures to biological outcomes for risk assessment purposes, such data are very valuable. The application of this information to human health or epidemiological studies has been limited [27] but have provided valuable insights into populations at special risk.

Mechanistic Linkages

Interpretation of proteomic biomarkers with regard to mechanistic linkages to mechanisms of cell injury/cell death or cancer are more easily interpreted since the biochemical function(s) of many proteins are well known. For example, the generally protective roles of stress proteins or metallothionein in attenuating cell injury are well known and may be factored into predictions of susceptibility to chemical injury. In addition, these proteomic responses are also reflective of mechanisms of toxicity (e.g., reactive oxygen species, ROS) by indicating which cellular pathways are responding to a given chemical exposure. Proteotoxicity (oxidation of proteins) indicative of ROS will result in upregulation of major stress protein classes as an early protective cellular response and will help to focus further studies on sensitive target protein classes. In addition, if the protective stress protein responses are attenuated due to a specific chemical, dose or duration of exposure [18] and linked to increased cellular toxicity (e.g., proteinuria), this is also valuable information in terms of predicting the limits of protection.

Risk Assessment Linkages

Proteomic biomarkers, as noted above, may be more directly useful than genomic biomarkers for risk assessment purposes since they represent translated gene products whose functions are frequently well understood at this point in time. This information means that the qualitative and quantitative nature of the proteomic response may provide more readily interpretable information for risk assessment purposes on likely health outcomes if the response persists with continued exposures. It should be noted that proteomic responses are finite and that depending upon factors such as the chemical agent involved, dose and duration of exposure, these responses may become attenuated and lead to overt cell injury [18]. As with genomic approaches, protein–protein pathway analyses have also been under development [30].

Metabolomic/Metabonomic Biomarkers

The metabolomic/metabonomic biomarker approach uses a variety of analytical approaches including HPLC with fluorescence detection, NMR, and mass spectrometry to follow chemical-induced disturbances in metabolic pathways as reflected by metabolic substrates or products generated by those pathways [39–45]. Early studies focused on alterations of the heme biosynthetic pathway produced by exposure to lead [43], methyl mercury [2, 3], arsenic [4–6], and a number of chlorinated organic chemicals [42, 44, 45] have been successfully used for decades in both experimental, clinical, and epidemiological studies. Other studies have focused on measurements of metabolites from intermediary and drug metabolism [46–49]. The increases in presence or excretion of metabolic precursors or metabolic products in accessible matrices

in response to chemical or drug exposures adds another level of information on the nature of the interaction between these agents and sensitive metabolic pathways and potential roles of disturbances in these pathways and overt clinical diseases.

Mechanistic Linkages

At this stage in the evolution of metabolomic/metabonomic biomarkers, there is a mixed picture where for some of these endpoints such as disturbances in the heme biosynthetic pathway, extensive research over decades has provided a wealth of information on linkages between disturbances in this pathway are linked to mechanisms of cell injury and cell death [50]. Another useful metabolite for improving the accuracy of estimates of smoking status is the nicotine metabolite cotinine which has been successfully applied to a number of epidemiology studies [51, 52] as a quantitative index of exposure.

For other pathways whose metabolic substrates or products are less well understood, further research is needed to both understand links to mechanisms of cellular toxicity but also for their incorporation into mode of action risk assessment strategies. Examples of these putative biomarkers would include products of metabolized chemicals or drugs which are excreted in the urine [42, 49] and alterations in serum or urine levels of normal intermediates in sensitive pathways such as the tricarboxylic acid (TCA) cycle [46].

Risk Assessment Linkages

As noted above, some metabolomic/metabonomic biomarkers such as those in the heme biosynthetic pathway have been successfully used in risk assessment practice as well-understood endpoints for predicting likely toxic outcomes from chemical exposures, developing lowest observable adverse effect levels (LOAELs) for exposure to toxic agents such as lead [53]. Delineated biomarkers in this class require further research to understand their prognostic significance and utility for incorporation into risk assessment strategies.

New Challenges

Mixtures

The issue of chemical mixtures is a common situation and of growing toxicological concern. Early studies [54, 55] utilized metabolomic disturbances in heme biosynthetic pathway to follow *in vivo* responses to mixtures of lead, cadmium, and arsenic at

elevated (stressor) dose levels for 10 weeks in rats. More recent lead, cadmium, and arsenic interaction studies [56] conducted time course studies in rats at empirically determined lowest observed effect level (LOEL) doses at 30-, 90-, and 180-day time points. These studies found similar interactive effects between these toxic elements for heme biosynthetic pathway endpoints but noted the importance of duration of exposure in influencing these outcome measures. Overview reviews [57, 58] of the roles of biomarkers in the evaluation of metallic mixtures provide useful summaries of this literature. Other studies on the III–V semiconductors gallium arsenide and indium arsenide used both metabolomic disturbances in the heme biosynthetic pathway [59–62] and proteomic approaches [17, 18] to evaluate the cellular effects of these binary compounds as 3–5-μm particles or soluble salts and marked gender differences in response to these agents on an individual or binary exposure basis.

Nanomaterials

The growing issue of toxic risks associated with the packaging of toxic chemical into nanomaterials is the subject of intense interest [63]. The importance of nanomaterials from the perspective of toxicity is that they may greatly increase absorption of chemical constituents, thus raising the internalized dose, and they appear to be effective catalysts for surface chemistry-induced formation of ROS. The net effect of these properties would be to increase predicted cellular toxicity and risk of cancer. For these reasons, biomarker endpoints may prove to be of great value in the early detection of effects from these agents, particularly at low dose levels.

Further Research Translational Needs

Biomarker Validation Needs

It should be clear from the above discussion that the various classes of biomarkers are being effectively used for the prediction of specific developed disease outcomes and hold great promise for both early detection of preclinical effects of chemicals and drugs but that for the latter to occur, extensive validation studies are needed in order for them to reach their full predictive potential and be translated into chemical risk assessment practice. This means that focused and well-designed studies linking chemical/drug exposure to the response of a particular biomarker or a suite of biomarkers to other health endpoints must be conducted to address the question of whether the biomarker response is simply a transitory event of no particular health significance or whether the response is a harbinger of adverse health events such as target organ toxicity, birth defects, or cancer. Such interpretive studies are arduous but can be done if sufficient resources are available. For example, Bayesian approaches [64] have been successfully

applied to validation of biomarkers for benzene exposure for which a rich database was available. Results of these studies demonstrate that while the issue of limited resources for biomarker validation is problematic, it should not impede progress in this field over the long term since the potential payoffs are enormous. On the other hand, in the absence of a robust biomarker database, the question of what types of studies are needed to validate evolving biomarkers must be considered [47, 49].

Bioinformatics

Another major issue confronting the incorporation of biomarkers into risk assessment practice is the fact that "omics" biomarkers generate large quantities of data which must be digested and interpreted in order for them to be fully appreciated. This means that appropriate bioinformatics tools must also be concomitantly developed to process large biomarker datasets into useable/understandable findings. It is also important for attention to be given to translating the results of bioinformatic findings into lay language so that persons with less technical backgrounds may better appreciate the practical implications of the findings [38, 65].

Computational Toxicology Modeling

In recent years, the application of computational modeling methods has expanded into a number of areas of toxicology and risk assessment. These modern methods have greatly improved the ability to rapidly focus resources on linkage between chemical exposures such as persistent organic pollutants (POPs) and important clinical diseases such as type II diabetes [29] via pathway and network analyses. In doing so, these methods have the capacity to help focus further biomarker studies on underlying causes of clinical diseases and the putative linkages between exposure to chemicals and health outcomes. Such information could be of great value in managing exposures to chemicals. In addition to the bioinformatics aspects noted above, these methods may also help to provide greater interpretive power to generated biomarker data and further the science by generating new hypotheses.

Translation of Biomarkers into Risk Assessment Practice: Changing the Paradigm: The Way Out of the Box

Based upon the brief summary above, it should be clear that molecular biomarkers are currently being effectively used in a number of clinical situations to monitor and predict the outcomes of developed diseases and that they have great potential for helping risk

assessment practice if a number of impediments can be addressed. The first of these is the need for complimentary validation studies noted above which are needed in order to permit correct interpretation of a given biomarker or suite of biomarkers in the scientific sense. Secondly, there is a need to reduce the frequently generated large quantities of biomarker data into interpretable components through the application of bioinformatics approaches. Thirdly, there is a very real need to translate the findings of biomarker studies into less technical terminology so that persons with diverse backgrounds can appreciate, incorporate, and apply these findings into other research fields such as epidemiology. In other words, it is very important to translate biomarker information into "plain English" so that this information may gain wider acceptance through improved understanding. This is important so that biomarkers gain overall acceptance in the scientific community in the long term. The integration of biomarker endpoints into epidemiology (e.g., molecular epidemiology) should be of particular value in providing a more sensitive and specific set of endpoints to help understand/interpret the results of traditional epidemiological studies on populations exposed to chemicals on an individual or mixture basis. As noted above, the further integration of computational modeling methods into such studies should increase the public health impact of biomarker measurements and advance the science in this increasing important area.

For the reasons noted above, there is considerable reason for optimism that biomarkers may yet reach their full potential as greatly needed tools for advancing science and risk assessment practice related to the chemical safety. Such information should greatly improve the sensitivity, precision, and accuracy of scientific judgments related to chemical exposures on an individual or mixture basis.

References

1. Fowler BA (1980) Ultrastructural morphometric/biochemical assessment of cellular toxicity. In: Witschi HP (ed) Proceedings of the symposium on the "scientific basis of toxicity assessment". Elsevier, New York, pp 211–218
2. Woods JS, Fowler BA (1977) Renal porphyrinuria during chronic methyl mercury exposure. J Lab Clin Med 90:266–272
3. Fowler BA, Woods JS (1977) Ultrastructural and biochemical changes in renal mitochondria during chronic oral methyl mercury exposure: the relationship to renal function. Exp Mol Pathol 27:403–412
4. Woods JS, Fowler BA (1978) Altered regulation of mammalian hepatic heme biosynthesis and urinary porphyrin excretion during prolonged exposure to sodium arsenate. Toxicol Appl Pharmacol 43:361–371
5. Fowler BA, Woods JS (1979) The effects of prolonged oral arsenate exposure on liver mitochondria of mice: morphometric and biochemical studies. Toxicol Appl Pharmacol 50: 177–187
6. WHO (1993) Biomarkers and risk assessment: concepts and principles. International Programme on Chemical Safety (ICPS), Environmental Health Criteria 155, WHO, Geneva, Switzerland, pp 1–35. Online available at http://www.inchem.org/documents/ehc/ehc/ehc155.htm
7. WHO (2001) Biomarkers in risk assessment: validity and validation. International Programme on Chemical Safety (ICPS), Environmental Health Criteria 222, WHO, Geneva, Switzerland, pp 1–154. Online available at http://www.inchem.org/documents/ehc/ehc/ehc222.htm

8. Franco SS, Nardocci AC, Gunther WMR (2008) PAH biomarkers for human health risk assessment: a review of the state-of-the-art. Cad Saude Publica 34(Suppl 4):569–580
9. Bhattacharya K, Dopp E, Kakkar P, Jaffery FN, Schiffmann D, Jaurand MC, Rahman I, Rahman Q (2005) Biomarkers in risk assessment of asbestos exposure. Mutat Res 579:6–21
10. Kostrubiec M, Pruszczyk P, Bochowicz A, Pacho R, Szulc M, Kaczynska A, Styczynski G, Kuch-Wocial A, Abramczyk P, Bartoszewicz Z, Berent H, Kuczynska K (2005) Biomarker-based risk assessment model in acute pulmonary embolism. Eur Heart J 26:2166–2172
11. Heidecker B, Kasper EK, Wittstein IS, Champion HC, Breton E, Russell SD, Kittleson MM, Baughman KL, Hare JM (2008) Transcriptomic biomarker for individual risk assessment in new-onset heart failure. Circulation 118:238–246
12. Swenberg JA, Fryar-Tita E, Jeong YC, Boysen G, Starr T, Walker VE, Albertini RJ (2008) Biomarkers in toxicology and risk assessment: informing critical dose-response relationships. Chem Res Toxicol 21:253–265
13. Vlaanderen J, Moore LE, Smith MT, Lan Q, Zhang L, Skibola CF, Rothman N, Vermeulen R (2010) Application of omics technologies in occupational and environmental health research: current status and projects. Occup Environ Med 2:136–143
14. Hagger JA, Jones MB, Leonard DR, Owen R, Galloway TS (2006) Biomarkers and integrated environmental risk assessment: are there more questions than answers? Integr Environ Assess Manag 2:312–329
15. Ryan PB, Burke TA, Hubal EA, Cura JJ, McKone TE (2007) Using biomarkers to inform cumulative risk assessment. Environ Health Perspect 115:833–840
16. Robeson RH, Siegel AM, Dunckley T (2008) Genomic and proteomic biomarker discovery in neurological disease. Biomark Insights 3:73–86
17. Fowler BA, Conner EA, Yamauchi H (2005) Metabolomic and proteomic biomarkers for III-V semiconductors: chemical-specific porphyrinurias and proteinurias. Toxicol Appl Pharmacol 206:121–130
18. Fowler BA, Conner EA, Yamauchi H (2008) Proteomic and metabolomic biomarkers for III-V semiconductors: prospects for applications to nano-materials. Toxicol Appl Pharmacol 233: 110–115
19. Goering PL, Fowler BA (1985) Mechanisms of renal lead-binding protein protection against lead-inhibition of δ- aminolevulinic acid dehydratase. J Pharmacol Exp Ther 234:365–371
20. Goering PL, Mistry P, Fowler BA (1986) A high affinity lead-binding protein in brain attenuates lead inhibition of δ- aminolevulinic acid dehydratase: comparison with a renal lead-binding protein. J Pharmacol Exp Ther 237:220–225
21. Goering PL, Fowler BA (1987) Kidney zinc-thionein regulation of δ-aminolevulinic acid dehydratase inhibition by lead. Arch Biochem Biophys 253:48–55
22. Goering PL, Fowler BA (1987) Metal constitution of metallothionein influences inhibition of δ-aminolevulinic acid dehydratase (porphobilinogen synthase) by lead. Biochem J 245: 339–345
23. Quintanilla-Vega B, Smith DR, Kahng MW, Hernandez JM, Albores A, Fowler BA (1995) Lead-binding proteins in brain tissue of environmentally-lead exposed humans. Chem Biol Interact 98:193–209
24. Smith DR, Khang MW, Quintanilla-Vega B, Fowler BA (1998) High affinity renal lead-binding proteins in environmentally exposed humans. Chem Biol Interact 115:39–52
25. Oskarsson A, Fowler BA (1985) Effects of lead inclusion on subcellular distribution of lead in rat kidney: the relationship to mitochondrial function. Exp Mol Pathol 43:409–417
26. Scinicariello F, Yesupriya A, Chang MH, Fowler BA (2010) Modification by ALAD of the association between blood lead and blood pressure in the U.S. population: results from the third National Health and Nutrition Examination Survey. Environ Health Perspect 118: 259–264
27. Lu J, Jin T, Nordberg G, Nordberg M (2005) Metallothionein gene expression in peripheral lymphocytes and renal dysfunction in a population environmentally exposed to cadmium. Toxicol Appl Pharmacol 206:150–156

28. Esteller M (2007) Cancer epigenomics: DNA methylomes and histone modification maps. Nat Rev Genet 8:286–298
29. Ruiz P, Fowler BA, Mumtaz M (2010) Persistent organic pollutants and metabolic diseases: Quantitative structure activity relationship (QSAR), protein networks and pathway analysis. Toxicology and Risk Assessment Conference (TRAC 2010), Cincinnati, OH, April 26–29, 2010
30. Wilson B, Liotta LA, Petricoin E 3rd (2010) Monitoring proteins and protein networks using reverse phase protein arrays. Dis Markers 28:225–232
31. Risk MC, Lin DW (2009) New and novel markers for prostate cancer detection. Curr Urol Rep 3:179–186
32. Srinivas PR, Verma M, Zhao Y, Srivastava S (2002) Proteomics for cancer biomarker discovery. Clin Chem 48:1160–1169
33. Petricoin EF, Liotta LA (2004) Proteomic approaches in cancer risk and response assessment. Trends Mol Med 10:59–64
34. Zhang Z, Chan DW (2005) Cancer proteomics: in pursuit of "true" biomarker discovery. Cancer Epidemiol Biomarkers Prev 10:2283–2286
35. Hanash SM, Pitteri SJ, Faca VM (2008) Mining the plasma proteome for cancer biomarkers. Nature 452:571–579
36. Benninghoff AD (2007) Toxicoproteomics—the next step in the evolution of environmental biomarkers. Toxicol Sci 95:1–4
37. Stenvinkel P, Carrero JJ, Axelsson J, Lindholm B, Heimbürger O, Massy Z (2008) Emerging biomarkers for evaluating cardiovascular risk in the chronic kidney disease patient: how do new pieces fit into the uremic puzzle? Clin J Am Soc Nephrol 3:505–521
38. Fliser D, Novak J, Thongboonkerd V, Argiles A, Jankowski V, Girolami MA, Jankowski J, Mischak H (2007) Advances in urinary proteome analysis and biomarker discovery. J Am Soc Nephrol 18:1057–1071
39. Schnackenberg LK, Beger RD (2008) The role of metabolomic biomarkers in drug toxicity studies. Toxicol Mech Methods 18:301–311
40. Lin CY, Viant MR, Tjeerdema RS (2006) Metabolomics: methodologies and applications in the environmental sciences. J Pest Sci 3:245–251
41. Viant MR (2009) Applications of metabolomics to the environmental sciences. Metabolomics 5:1–2
42. Kim KB, Kim SH, Um SY, Chung MW, Oh JS, Jung SC, Kim TS, Moon HJ, Han SY, Oh HY, Lee BM, Choi KH (2009) Metabolomics approach to risk assessment: methoxyclor exposure in rats. J Toxicol Environ Health A 72:1352–1368
43. Oskarsson A, Fowler BA (1985) Effects of lead on the heme biosynthetic pathway in rat kidney. Exp Mol Pathol 43:397–408
44. Gorman N, Trask HW, Bement WJ, Szakacs JG, Elder GH, Balestra D, Jacobs NJ, Jacobs JM, Sinclair JF, Gerhard GS, Sinclair PR (2004) Genetic factors influence ethanol-induced uroporphyria in $Hfe^{(-/-)}$ mice. Hepatology 4:942–950
45. Smith AG, Elder GH (2010) Complex gene-chemical interactions: hepatic uroporphyria as a paradigm. Chem Res Toxicol 23:712–723
46. Nagai R, Brock JW, Blatnik M, Baatz JE, Bethard J, Walla MD, Thorpe SR, Baynes JW, Frizzell N (2007) Succination of protein thiols during adipocyte maturation: a biomarker of mitochondrial stress. J Biol Chem 282:34219–34228
47. Law W, Huang P, Ong ES, Sethi S, Saw S, Ong CN, Li S (2009) Combination of ^{1}H nuclear magnetic resonance spectroscopy and liquid chromatography/mass spectrometry with pattern recognition techniques for evaluation of metabolic profile associated with albuminuria. J Proteome Res 8:1828–1837
48. Guéraud F, Atalay M, Bresgen N, Cipak A, Eckl PM, Huc L, Jouanin I, Siems W, Uchida K (2010) Chemistry and biochemistry of lipid peroxidation products. Free Radic Res 44:1098–1124
49. Sobus JR, Pleil JD, McClean MD, Herrick RF, Rappaport SM (2010) Biomarker variance component estimation for exposure surrogate selection and toxicokinetic inference. Toxicol Lett 199:247–253

50. Silbergeld EK, Fowler BA (1987) Mechanisms of chemical-induced porphyrinopathies. Annals of the New York Academy of Sciences, New York
51. CDC (2010) Vital signs: nonsmokers' exposure to secondhand smoke—United States, 1999–2008. Centers for Disease Control and Prevention. MMWR Morb Mortal Wkly Rep 59:1141–1146
52. Benowitz NL, Dains KM, Dempsey D, Yu L, Jacob P 3rd (2010) Estimation of nicotine dose after low-level exposure using plasma and urine nicotine metabolites. Cancer Epidemiol Biomarkers Prev 19:1160–1166
53. Scinicariello F, Murray HE, Moffett DB, Abadin H, Sexton MJ, Fowler BA (2007) Lead and δ-aminolevulinic acid dehydratase polymorphism: where does it lead? a meta-analysis. Environ Health Perspect 115:35–41
54. Fowler BA, Mahaffey KR (1978) Interaction between lead, cadmium and arsenic in relation to porphyrin excretion patterns. Environ Health Perspect 25:87–90
55. Mahaffey KR, Capar SG, Gladen BC, Fowler BA (1981) Concurrent exposure to lead cadmium and arsenic: effects on toxicity and tissue metal concentrations in the rat. J Lab Clin Med 98:463–481
56. Whittaker MH, Wang G, Chen XQ, Lipsky M, Smith D, Gwiazda R, Fowler BA (2011) Exposure to Pb, Cd, and As mixtures potentiates the production of oxidative stress precursors: 30-day, 90-day, and 180-day drinking water studies in rats. Toxicol Appl Pharmacol 254: 154–166
57. Fowler BA, Whittaker MH, Lipsky M, Wang G, Chen XQ (2004) Oxidative stress induced by lead, cadmium and arsenic mixtures: 30-day, 90-day, and 180-day drinking water studies in rats: an overview. Biometals 17:567–568
58. Wang G, Fowler BA (2008) Roles of biomarkers in evaluating interactions among mixtures of lead, cadmium and arsenic. Toxicol Appl Pharmacol 233:92–99
59. Goering PL, Maronpot RR, Fowler BA (1988) Effect of intratracheal administration of gallium arsenide administration on δ-aminolevulinic acid dehydratase in rats: relationship to urinary excretion of aminolevulinic acid. Toxicol Appl Pharmacol 92:179–193
60. Conner EA, Yamauchi H, Fowler BA, Akkerman M (1993) Biological indicators for monitoring exposure/toxicity from III-V semiconductors. J Exp Anal Environ Epidemiol 3: 431–440
61. Conner EA, Yamauchi H, Fowler BA (1995) Alterations in the heme biosynthetic pathway from III-V semiconductor metal indium arsenide (InAs). Chem Biol Interact 96:273–285
62. Madden EF, Fowler BA (2000) Mechanisms of nephrotoxicity from metal combinations: a review. Drug Chem Toxicol 23:1–12
63. Fowler BA (2009) Monitoring of human populations for early markers of cadmium toxicity. Toxicol Appl Pharmacol 238:294–300
64. Hack CE, Haber LT, Maier A, Schulte P, Fowler B, Lotz WG, Savage RE (2010) A method for biomarker validation and biomarker-based dose-response: a case study with Bayesian network. Risk Anal 30:1037–1051
65. Griffiths WJ, Koal T, Wang Y, Kohl M, Enot DP, Deigner HP (2010) Targeted metabolomics for biomarker discovery. Angew Chem Int Ed Engl 49:5426–5445

A Personalized Life: Biomarker Monitoring from Cradle to Grave

Harald Jungnickel and Andreas Luch

Abstract Considering the holy grail of future medical treatment being personalized medicines, biomarker research will become more and more the focus for attention not only to develop new medical treatment regimes, based on changes in biomarker patterns, but also for nutritional advice to guarantee a lifelong optimized health condition. The current review gives an outline of how personalized medicine can become established for actual medical treatment using new biomarker concepts. Starting from the development of biomarker research using mainly immunological techniques, the review gives an overview about biomarkers of prediction evolved and focuses on new methodology for the identification of biomarkers using hyphenated analytical techniques like metabolomics and lipidomics. The actual use of multivariate statistical methods in combination with metabolomics and lipidomics is discussed not only for medical treatment but also for precautionary risk identification in human biomonitoring studies.

Keywords Biomarkers · Biosensors · Imaging techniques · Biomarkers of prediction · Metabolomics · Metabolic fingerprints · Lipidomics · Shotgun lipidomics · High-throughput platforms · Personalized medicine · Human biomonitoring · Precautionary medicine

H. Jungnickel (✉) and A. Luch (✉)
Department of Product Safety, German Federal Institute for Risk Assessment (BfR), Max-Dohrn-Strasse 8-10, 10589 Berlin, Gemany
e-mail: Harald.Jungnickel@bfr.bund.de; Andreas.Luch@bfr.bund.de

A. Luch (ed.), *Molecular, Clinical and Environmental Toxicology*,
Experientia Supplementum 101, DOI 10.1007/978-3-7643-8340-4_17,

Introduction

Health and medical treatment as we know them nowadays may change significantly during the next decades. Today, we mostly stick to the paradigm of medical treatment for already physically established diseases based on the analysis of disease-related diagnostic "late-stage" parameters becoming visible only after disease onset [1]. In the future, early diagnostic measures may develop into a medical tool to prevent a wide range of illnesses already from the very beginning or even avoid them through regulatory interferences initiated right after early detection of biomarker disturbances far before any actual disease onset could be diagnosed at present [2]. Monitoring various parameters from childhood or even better already from the fetal stage onward over the whole lifespan of individuals may change the view we see and experience medicine as a whole. A combination of genetic, proteomic, and metabolic profiles together with biomarker development and multivariate statistical methods is likely to become a significant tool in future health care and medicine. The actual prophylaxis of diseases may be supported by the re-adjustment of aberrant biomarker patterns back to normality. Diseases, toxic environmental influences, unhealthy diet, and genetic predisposition as well as a mutual interaction of all of them may become treatable only if really needed and with much lower drug dose regimens as with up-to-date general precautionary treatment arrangements. The development of new or the use of existing sets of biomarkers is likely to become a paramount theme for research to fulfill the requirements of preventive and personalized medicine.

With the advent of immunological testing, the heydays of molecular biomarkers started [3]. For a biomarker to be useful in diagnostics, it must be easily accessible to analytical detection, meaning its presence in body fluids, preferably urine or blood, in above-normal levels in comparison to healthy humans. It has to be as specific as possible to one disease to avoid "wrong positive" diagnosis. The biomarker should be stable over time in the medium from which it is extracted, at least at storage temperatures ($-20°C$ or $-80°C$), because, in most epidemiologic studies, immediate laboratory processing is not available. Ideally, a valuable biomarker should reflect the extent and the spread of the disease with a significant change in concentration during disease progression.

One of the first used enzymes in clinical enzymology was human chorionic gonadotropin (HCG) [4]. It was not only used for pregnancy testing but also for the diagnosis of placental cancer. Elevated levels of HCG could be used for tumor diagnosis because some kinds of tumor cells permanently produce it. Proteins like HCG were almost exclusively used as biomarkers in the early days. In general, established biomarkers usually became instrumental in the detection of matured diseases but suffered from the handicap that early disease stages remained undetected. Nevertheless, protein biomarkers were a success story due to the comparable easy, cheap, and fast testing regime: the blood test. The introduction of radioimmunoassays (RIA) facilitated the use of a variety of different protein-based biomarkers in blood. Insulin was the first enzyme measured regularly by RIA [5].

RIA facilitated—among others—the detection of human rectum and colon cancer [6], prostate cancer [7], neuroendocrine tumors [8], systemic iron homeostasis disturbances [9], and circadian dysregulation [10]. Many RIA were later on replaced by enzyme-linked immunosorbent assays (ELISA), using colorimetrical detection instead of radioactive labeling and detection.

Some of the early protein biomarkers are still among the gold standards nowadays, like elevated serum creatinine levels for the diagnosis of acute kidney injury [11]. However, most of them have underwent significant changes over the past decades, mostly due to introduction of new and more sensitive analytical instrumentation becoming available to biomedical sciences and biomarker identification. If we have a closer look at the biomarkers for myocardial infarction, we actually observe a change of the biomarkers used to diagnose heart disease with almost every decade. All started in 1954, when LaDue *et al*. detected elevated glutamic oxaloacetic transaminase levels in human patients with myocardial infarction [12]. Over the years and with the detection of more specific biomarkers, glutamic oxaloacetic transaminase was first replaced by lactate dehydrogenase (LDH) [13] and creatine kinase (CK) [14]. In 1964, Burger *et al*. [15] proved the presence of three main cytoplasmic isoenzymes of CK: MM, BB, and MB with M and B being the monomer subunits. In 1972, Konttinen and Somer showed that serum CK MB levels are directly correlated to human infarction [16], and Roberts *et al*. proved in 1975 that it even can be used as an estimate for infarct intensity [17]. Since 1989, troponin was used to diagnose myocardial infarction because it proved more specific and sensitive to heart tissue injury than LDH and CK [18]. At first, troponin T was used and succeeded at the beginning of the twenty-first century by troponin I. All the above-mentioned biomarkers have in common that they can only be used to diagnose myocardial infarction but are of no value for infarct risk assessment prior to the onset of the actual ischemic event.

Nowadays, imaging techniques and multicomponent biomarker systems are in development to assess infarct risks well before the actual infarction takes place, leading to “biomarkers of prediction” rather than “biomarkers of disease states” [19]. The great benefits of imaging techniques are the visualization of biochemical distributions in biological tissues for interpretation of, for instance, derailed metabolism initiated during pathogenesis or triggered by compound-mediated toxicological effects. A set of biomarkers usable to predict infarction would open the door to an efficient preinfarct medication, but similar efforts may also lead to an intelligent personalized medical treatment of other diseases and even pave the road for precautionary measures. As an encouraging perspective, new imaging techniques even capable of 3D imaging of single cells with high spatial resolution are currently being established [20]. In combination with new statistical approaches [21], such techniques could ultimately result in a complete new “virtual” assessment of biochemical distributions far down to the single-cell level. Subcellular distributions could be uncovered, and the knowledge on actual enrichment sites of biosynthetic end products or extracellular pollutants would provide valuable information and helpful guide in health risk assessments.

Biomarkers for Prediction

In 1976, Kannel *et al*. developed the first multivariate risk prediction model, introducing a statistical method to evaluate a set of biomarkers for risk prediction in cardiac disease [22]. Having a set of biomarkers available for risk prediction also implies the need for appropriate methods to evaluate and validate newly emerging biomarkers to make decisions whether to include or exclude them from already existing models.

Looking at the development of new biomarkers directly prompts the question: Why is a new biomarker needed and how can it be assessed and validated against existing biomarkers? Ideally, the biomarker discriminates between healthy (negative) and diseased (positive) patients. A biomarker also has to fulfill the two criteria: "specificity" and "sensitivity." While specificity means people being falsely identified as positive using the biomarker (e.g., after biomarker measurement a patient is diagnosed with myocardial infarction but did not actually have one), sensitivity means falsely negatively identified people (e.g., patients will be diagnosed as healthy but have had actually myocardial infarction). Ideally, both specificity and sensitivity are 100%, but this is never achievable in reality. Therefore, all existing biomarkers compromise either specificity or sensitivity or both. Newly developed biomarkers have to be assessed against existing ones, and a conclusion has to be drawn whether the use of the new biomarker adds additional information to the existing diagnosis regime. D'Agostino [23] describes four decision criteria for the evaluation of a new biomarker. Besides a general definition of the experimental context (for which group of people the biomarker shall be used, what diagnostic result is anticipated, how preexisting biomarkers should be incorporated in a new diagnostic regime), the question which model is the appropriate one to evaluate the added value of the new biomarker becomes central. According to this, a statistically significant correlation of a biomarker with a given disease is not enough to implement it into the diagnostic regime; instead, it has to prove that it actually adds additional value to existing diagnostics. One of the key questions therefore is which model is most appropriate for biomarker assessment and how to best assess a new biomarker against the existing ones.

In the past, a new biomarker was accepted when it actually improved the "area under the curve" (AUC) in the receiver operating characteristic curve [24]. The AUC index is defined as the probability that in a randomly selected pair of healthy and diseased patients, the individual biomarker or a set of biomarkers will indicate the diseased patient. It is expressed as probability value ranging from 0 to 1, where values close to 1 indicate high diagnostic accuracy. One disadvantage of the AUC model is that for models containing standard risk factors combined with a reasonably good discrimination, a high correlation of the biomarker with the anticipated outcome is required to achieve an acceptable increase in the AUC and therefore accept the new biomarker as a tool worthy to be incorporated in the already existing biomarker testing strategy [25]. Therefore, recently, new strategies evolved concentrating on the improvement of the used model rather than waiting for

new biomarkers, which could attribute to existing models [26]. The new models concentrate on two topics: either redistributing patients into new smaller risk groups or developing new models for prediction. With the redistribution of patients into smaller subgroups, the probability of a new biomarker to add value to the existing model increases significantly [27]. If, for example, the level of a given biomarker changes not only with the disease under investigation but can also be correlated with another malignancy, the falsely positive identified patients may increase significantly, therefore reducing the added AUC gain. All patients suffering from the malignancy not under consideration will show up as false positives. If two groups are established, one containing patients who suffer from the malignancy and the other consisting of patients not suffering from it (but who suffer from the actual disease under investigation), the new biomarker may well add additional discriminative power to the model but only within the group of patients who do not suffer from the malignancy. In that way, new biomarkers may well even replace older already established biomarkers but only in specific subgroups. As can be seen from the example above, choosing the right subgroups is the most important criteria for success [27]. And a specific set of subgroups would have to be defined for each biomarker application. One way would be to investigate the false positively grouped patients and the falsely negative grouped ones to find criteria which fit for all of them, so that they could be excluded as a subgroup. The challenge with this method is that an as-small-as-possible number of patients from the subgroup, for which the biomarker actually will be finally applicable, should be grouped into the excluded subgroup using the identified new criteria to set up the new subgroups. As disadvantage of this approach, a multitude of subgroups may emerge, and—for correct subgrouping—a variety of biochemical and/or assessment factors need to be collected, thereby significantly increasing the costs for each test.

On the other hand, multivariate statistical analysis in combination with machine learning networks might be a more promising tool to base health prediction and risk assessment not only on single biomarkers but rather on a set of intertwining metabolic actions which define a "biomarker space" for decision making, for example, by using integromics features [28]. To this end, correct subgrouping of patients or populations, in combination with advanced statistical data treatment, is needed to further develop robust scientific models for the evaluation of data gained via human biomonitoring studies and/or genomic, proteomic, and metabolomic analyses. Multivariate statistical data procedures and neuronal network statistics are needed to evaluate the resulting datasets and to get a more individualized outcome, which then hopefully could be applied and directed toward a better disease treatment.

Metabolomics in Biomarker Research

Metabolomics is a new emerging field in biomarker research [29]. Based on the assumption that diseases as well as therapeutic interferences, drug intake, stress factors, inflammation, and even environmental chemicals can be traced using

physiological information from the metabolome, the changes in metabolite levels can be used for biomarker research and identification. A great advantage of metabolomics is the fact that early disease stages in patients, which cannot be diagnosed by the use of conventional methods, usually are already manifest in significant metabolic profile changes and in the generation of specific metabolic fingerprints [30]. Additionally, the metabolic profile might be re-adjustable by intervention, meaning it can be changed from a "diseased status" back to a "healthy status" once the patient really recovers [31]. If the changes will be continuous, medical intervention might be judged effective or ineffective a long time before actual physical and/or physiological improvements are visible. Metabolomics therefore is expected being a valuable tool in assessing humans at risk not only for disease development but also for early stage decision making in health-care intervention and medical treatment.

Studying the metabolic profiles of urine, blood, saliva, or even individual organs and tissues may yield important information about the actual physiological condition of the patient [32]. However, especially protein levels in urine, blood, and saliva remain challenging due to the wide concentration ranges of proteins in these samples. New developments in analytical technology, especially in the areas of mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy, have initiated a low-level quantification approach for a multitude of metabolites targeted by medical and risk assessment biomarker research [33].

Usually, different approaches are needed to collect as-much-as-possible metabolic information, for instance, by combining gas chromatography (GC)–MS, liquid chromatography (LC)–MS, and NMR datasets [34, 35]. Additional information can be obtained by linking the gathered metabolomic information with proteomics and genomics data, thereby generating a framework from gene expression levels, protein activities, and metabolome alterations [36]. Statistical tools are then applied to identify possible sets of biomarkers either for risk prediction or medical treatment. Ideally, an interlinking biomarker approach can be used, combining not only biomarkers from microarray genomic data but also proteomic data and metabolomic data in an integrated biomarker set [36]. The identification of such integrated biomarker sets in the future is expected to lead to new insights into disease initiation, progression, and medical counteraction. Even more, such a metabolic approach will open up completely new ways in disease prevention based on lifelong individual metabolic profiling. However, one difficulty in metabolomics is to actually establish meaningful subgroups like healthy and diseased patients based on metabolic profiles. Since metabolic profiles are expected to change already at extremely early disease states, which may not be detectable by regular medical diagnostics, very easily, an already deranged metabolic profile could be judged "healthy" using currently available practices and thus falsify the results [37]. This clearly shows that much more additional information, especially data comprising early and extended time periods in life, is needed to decide whether metabolic profiles actually belong to the "healthy" or to the "ill" group. One approach would be a complete metabolic profiling from childhood to old age. This almost certainly would guarantee to detect even diminutive changes in metabolic profiles as early as possible [38].

Table 1 Targeted *versus* untargeted metabolomics

	Targeted metabolomics	Untargeted metabolomics
Analytes	Predefined set of metabolites from selected metabolic pathways	Qualitative analysis of "as-much-as-possible" metabolites
Information depth	Full quantification, authentic reference compounds required	Qualitative high-throughput metabolic fingerprinting of full chemical space
Instrumentation	LC–MS/MS (MRM) GC–MS/MS (MRM)	LC–MS/MS accurate mass GC–MS/MS accurate mass
Analytical method	Target ions analyzed	All recorded ions analyzed
Outcome	Exact quantification of selected metabolites from defined metabolic pathways	Enables metabolic fingerprinting and identifies so far unknown metabolite changes in various metabolic pathways

Targeted and Untargeted Metabolomics

Current metabolomic research focuses on two different approaches, that is, targeted and untargeted metabolomics (Table 1) [39]. The targeted metabolomics approach tries to focus on a certain set of metabolites rather than trying to quantify all metabolites available in the sample. Multiple reaction monitoring (MRM) using tandem quadrupole mass spectrometry is predominantly applied in this kind of approach [40]. MRM identifies the molecular ion of a particular metabolite and uses its first fragment ion for compound identification. This approach proved especially useful for high-throughput analysis, which is hard to achieve using an untargeted metabolomic approach [41]. The use of MRM allows for the absolute quantification of individual metabolites with high sensitivity and reproducibility. Usually, the targeted metabolites are chosen from different metabolic pathways, ideally identified beforehand in nontargeted metabolomic approaches as being variable in disease progression, drug treatment arrangements, and quality control groups [42]. After metabolite identification, reference compounds are analyzed, thereby optimizing the conditions for the molecular ion by varying polarity and cone voltage and for the fragment ion by polarity switching and collision energy. After condition optimization, high-throughput analysis is performed using the obtained conditions with 96-well microtiter plates in combination with ultra performance liquid chromatography (UPLC) or Fast LC analysis [43]. Especially the development of UPLC and Fast LC techniques enables fast analysis time, normally being less than 10 min for one single run, combined with high performance liquid chromatography (HPLC) separation for more accurate metabolite quantification. Usually, LC–MS/MS methods are validated for regulatory purposes [44]. Either Flow Injection Analysis or LC separation is used on the front end. LC is used to separate compounds before analysis to reduce observed analyte suppression and to resolve isomeric and chiral compounds before quantification. The MRM experiments have to be optimized for maximum sensitivity, reliable quantification, and the reduction of false positives. Quantitation has to be validated within the method. Limits of detection (LOD) and limits of quantification (LOQ) must be established for each analyte. Standard stability and reagent stability studies have to

be performed for all solutions used in the method. Day-to-day reproducibility of standard curves and sample repeats have to be analyzed, and the accuracy of the method has to be determined.

Currently targeted metabolomics is used for quality control of biological samples and for most human biomonitoring studies. One example is the quality control of green tea using NMR-based metabolomics [45]. In addition, LC–UV or LC–MS/MS methods were applied in quality control of herbal extracts [46]. Using multivariate analysis for data interpretation allows to group these extracts for quality control purposes, based on a set of marker compounds. Although herbal extracts are as yet not regulated by the US Food and Drug Administration (US FDA) or the European Pharmacopeia similar as pharmaceutical drugs are, the increasing use of such sources for medicinal remedies implies an urgent effort for the regulation of quality control procedures in the production of herbal medicines [47]. Another interesting approach for food quality control is the application of biological sensors. It has been shown that ethylene and acetaldehyde release can be used to monitor the quality of citrus fruits [48]. The chemical *in situ* sensor consists of a mammalian cell line, which is genetically engineered for the consecutive expression of the biosensor. The biosensor originally represents a gene of *Aspergillus nidulans* and triggers quantitative reporter gene expression in the presence of volatile acetaldehyde. Thinkable for the future is the combination of a multitude of biosensors, which can be used not only for volatiles but also for metabolites present in liquid matrices including sweat to continuously monitor online metabolomic changes, for instance, in body fluids or in human breath [49]. Using portable analytical inhaling equipment in combination with the development of threshold levels for volatile biomarkers could result in a modern lifestyle, where medical advice could be based on volatile personalized profiles. This again would also open up the opportunity for early stage detection of aberrant metabolic profiles. In that way, time profiles from individual human beings over a longer time period might be accessible, suitable to indicate rapid metabolomic changes directly *in situ* and thus alerting to seek health care or medical advice.

The second approach, that is, untargeted metabolomics, tries to use all available information from the global metabolome [50]. The idea is to analyze as many as possible metabolites and to monitor their changes; mostly relative quantification is applied. Untargeted metabolomics can be used, for instance, to provide insights into the alteration of metabolite levels and their accumulation in mutant strains in comparison to wild-type strains. For instance, the use of mass spectrometry and multivariate statistics of wild type *versus* methionine overaccumulation 1 (*mto1*) or transparent *testa4* (*tt4*) mutant strains in *Arabidopsis thaliana* resulted in a set of up- and downregulated metabolites, which allowed to discriminate between wild-type and mutant strains [51]. Some 34 compounds were identified as to be significantly different between *mto1* and the wild-type strain, and 31 compounds served to separate *tt4* from the wild-type strain. In *mto1*, significant accumulation of methionine, homocysteine, and methionine sulfone were observed, which correlates well with the methionine-accumulation pathway. Interestingly, metabolite changes in nontarget pathways can also be observed indicating coregulation of otherwise unlinked metabolic pathways in the genome [51]. Therefore, the comparison of

mutant strains *versus* wild-type strains allows significant insights into the genomic correlation of metabolic pathways and in resulting up- or downregulation of cellular metabolite levels. Similarly, overexpression of single genes can yield significant insights into changed metabolic patterns, when compared to wild-type strains. Both methods can be used in the future for "compromised individual testing."

Recent developments in accurate mass spectrometry help to advance the untargeted metabolomics approach significantly [52]. Untargeted metabolomics results in a set of unknown marker compounds, which can be used to discriminate between different datasets or treatment arrangements. The main task is to identify these unknown biomarkers in the following. Only with accurate compound identification a correlation to up- or downregulated metabolomic pathways becomes feasible. However, usually a purification step is needed to yield the pure compounds under investigation, which then are being analyzed with accurate mass spectrometry and NMR spectroscopy to ultimately identify their chemical entity [50]. Very often, only trace amounts of a particular compound are available for identification in metabolomics. Recent advances in instrument development however have opened up new possibilities to identify metabolites even from tiny fractions [53]. Combining high scanning rates with high mass accuracy for molecular ions and fragment ions is essential for this approach. Using new approaches like the molecular mass formula generator from Bruker allows for the first time advanced metabolite identification based on isotope patterns combined with statistical approaches [54]. Intelligent software automatically suggests the selection of the right molecule and its possible fragmentation pattern. After metabolite identification and relative quantification, using internal standards, data interpretation is mostly performed by multivariate statistical data interpretation. Therefore, the need for advanced metabolic software is obvious. A variety of different suppliers focuses on the distribution of metabolic package software, automatically using peak retention time alignment features, mass spectral identification libraries, and multivariate statistical tools to automatically extract biomarker candidate molecules [55]. Future advanced approaches will probably focus on multicomponent biomarker sets to diagnose disease and disease treatment or to predict nontoxic environmental chemical doses.

Since hardly any facility has the possibility of conducting targeted and untargeted metabolomics, various laboratories have developed specific sets of metabolites, which cover key metabolic routes from different metabolic pathways (lipid metabolism, glycolysis, *etc*.) [56]. Also limited metabolomic kits are available on the market, which try to specifically cover key metabolites from major metabolomic pathways.

Some Fields of Application and Expectations

Metabolomics is also used to enhance positive outcomes of *in vitro* fertilization, using a noninvasive method to determine embryo viability based on metabolite biomarker profiling of oocytes, blastocytes, and embryos [57]. The embryo development is associated with specific metabolic changes that can be analyzed directly

and noninvasively from the culture medium of *in vitro* fertilized eggs [57]. Fingerprint metabolite patterns can be used as biomarkers for the prediction of embryo viability and thus may ultimately be helpful to increase *in vitro* fertilization success rates. Given that different chemical fingerprints can be observed from morphological similar embryos, the metabolic fingerprint approach clearly would be expected superior to the currently used visible inspection-only approach [57].

The investigation of prolonged time line sequences, for instance, to monitor disease regression after drug treatment or the metabolic profile changes upon exposure to sublethal doses of environmental chemicals, necessarily entails the possibility for analyzing larger sample sets [58]. New instrumentation is needed to cope with large sample sets for mass spectrometry and NMR spectroscopy. Robotics subsequently emerged as a new research field, using so-called automated sample preparation platforms [59]. Included are all sample pretreatment steps prior to mass spectral analysis. Semiautomated systems deal with all pipetting tasks, vacuum-based solid phase extraction steps for high-throughput analysis, designed for microtiter well plates, incubation steps, sample drying and centrifugation, and sample derivatization steps, if needed [60]. The resulting samples are ready to be injected into the mass or NMR spectrometer. Nowadays, validated sample protocols are available for use in regulated GMP environments with full documentation, sample tracking, audit, and reporting capability [61]. High-throughput sample preparation is achieved by the use of 1,536-well pipetting devices to enhance sample throughput and guarantee efficient serial dilution for metabolomics studies [62]. Combined with faster analysis times (less than 5 min for one run), new high-throughput platforms capable of analyzing several hundred samples a day will emerge. For that purpose, miniaturization of sample preparation and analyte separation methods are necessary. Recent developments focus on microcapillary electrophoresis, Lab-on-a-chip, and nano-HPLC devices [63, 64]. New software development allows for 3D simulation of the chosen sample pretreatment steps prior to actual implementation, a task helpful to avoid instrumental errors resulting from wrong programming steps [65].

Especially for NMR quantification, online sample preparation combined with LC methods is needed to cope with the complexity of samples that contain the entire metabolomes of cells [66]. Some groups even try to develop a combined metabolomic platform, capable of analyzing samples with LC–MS/MS and NMR (cf. below). In line with this direction, Sun *et al*. showed in a metabolomics study in 2010 that the combination of NMR and LC–MS data can be used to identify biomarkers in the metabolome of urine samples from rats after acrylamide application (14-day dosing) [67]. Acrylamide causes genotoxicity, neurotoxicity, and defects of reproduction in rodents and is considered as carcinogen and neurotoxin in humans [68]. Using LC–MS/MS analyses, three mercapturic acid conjugates of acrylamide were detected via exact mass and principal component analysis (PCA) of urine samples. In addition, NMR analyses revealed an increase in creatine and a decrease in taurine levels. The overall results suggest that tricarboxylic acid cycle (TCA) metabolites were downregulated later in the dosing period. Furthermore, many amino acids were upregulated during treatment and related to the weight loss observed in this study [67]. The encouraging outcome of this and related studies may ultimately lead to comparable human metabolome studies, from blood

samples, urine samples, or even microbiopsies (tissue specimens). These may then enable to predict early onsets of various diseases or to detect metabolome alterations indicative for human exposures against toxic environmental chemicals [69].

Modern medicine may even go a step further using metabolomics data. If metabolomic fingerprints for a variety of different diseases and toxic environmental influences are available, the changes in a specific metabolic profile over a given time period may not only indicate early disease onset. If the positive effects, caused by pharmaceuticals, herbs, or even specific food items, are known, a targeted approach may become thinkable where these sources are used to modify an observed aberrant metabolomic profile back to its healthy state or "normal" metabolomic profile [70]. In that way, a lifetime metabolomic profiling combined with a new approach in preventive health care made available routinely to everybody may significantly enhance the quality of life and help to prolong life spans even further [71]. Diseases may be treated on the very onset or outbreaks, and genetic disorders may be counteracted by pharmaceuticals and drugs while being closely monitored at the metabolomics level. Given this notion, it may not even be irrevocable that genetic disorders always ultimately lead to phenotypic manifestation. Metabolomics may develop into a tool that helps to reliably assess whether a genetic precondition may come into effect on the individual basis. There may be the possibility to prompt the initiation of health-care measures based on a negative metabolic profile change and the reaching of certain threshold levels for specific marker compounds. If a threshold concentration of a specific marker compound is observed, specific counteracting medical treatments, comprising—among others—pharmaceuticals, herbs, or food ingredients, may be initiated. In that way, even drug doses might be significantly reduced, when accompanied by cotreatment with herbs or specific food items.

Complex Metabolomic Platforms

Anticipated is the combined information from GC–MS/MS, LC–MS/MS, and LC–NMR data in one integrated software platform that can deal with large datasets and that is capable of interpreting acquired data using multivariate data analysis [50]. A combined dataset of GC–MS and LC–MS/MS nontargeted metabolomic profiles and targeted LC–MS analysis of total carbohydrates, monosaccharides, proteins, and biogenic amines, along with multivariate statistical analysis, was used in quality control to discriminate between 21 different coffee blends [72]. The method was also used to separate different coffee production sites. A 3D PCA plotting was used to visualize the results and revealed that the method could successfully discriminate between African, South American, and Asian blends. Another advantage of metabolomics is that pattern recognition algorithms can be utilized for the grouping of unknown samples [73].

What looks like a great opportunity, the variety of different data processing platforms available on the market from different technical suppliers, might also turn into an obstacle since data conversion between platforms is not always easy and may even result in the loss of important information from raw data [74].

At the end, however, a set of biomarkers will emerge from each experiment indicating major validated characteristic compounds, responsible for group separation. The developed methods should ideally cover all possible metabolomic targets including human blood, human serum, plant cells as well as microbial cells, and tissue cultures.

Lipidomics in Biomarker Research

Lipidomics is another approach to investigate more specialized metabolomic data available from either MS or NMR analysis [75]. Focusing on the lipid metabolic pathways, lipidomics not only investigates the storage of chemical energy and the changes in the outer cell membrane phospholipid patterns but also examines changes in (intra)cellular levels of, for instance, second messenger molecules from the groups of eicosanoids, prostaglandins and diacylglycerols, fatty acids like arachidonic acid or eicosapentaenoic acid, lysophospholipids, or inositol 1,4,5-triphosphate (IP_3), and of the broad field of steroids and sterols (Table 2). Lipidomics needs an analytical step in the first instance, yielding either qualitative or quantitative information about lipid molecules. Preferably, MS-based techniques are used nowadays [76]. With the advent of high scanning time-of-flight (ToF) mass spectrometers in combination with high mass accuracy, a rapid methodology now became available to detect and identify a broad variety of lipid molecules from complex biological matrices without extensive sample pretreatment [53]. Additionally, fast liquid chromatographic techniques, like UPLC or Fast LC, allow a significant reduction in sample acquisition time, bridging the way to huge datasets comprising not only from a multitude of single acquisitions but also from a high number of data points available for each single run. Modern statistical multivariate tools enable a fast, automated data mining approach, directly after sample acquisition [43]. If combined with multivariate statistical data reduction tools, lipidomics may be well suited to yield a variety of different biomarker sets for various diseases and genetic preconditions and—what is even more promising—to establish early onsets of disease states and possible adverse effects resulting from environmental exposures to hazardous chemicals [77].

Table 2 Principles, required instrumentation, and targets of lipidomics

	Shotgun lipidomics	LC–MS/MS-based lipidomics
Instrumentation	Quadrupole MS/MS	Quadrupole MS/MS
Inlet	Chip-based nanospray source	UPLC or Fast LC
Target molecules	Phospholipids from cell membranes, fatty acid metabolism	Phospholipids from cell membranes, fatty acid metabolism
Outcome	Exact quantification of selected metabolites from defined metabolic pathways combined with high-throughput analysis; no preseparation required	Exact quantification of selected metabolites in combination with fast separation step

Lipidomics also tries to identify possible enzymes involved in aberrant lipid patterns together with the identification of associated up- or downregulation of genes [78]. A complex picture of the real activity pattern of whole organisms down to single cells may become available after combining with data from the genome, the proteome, and the metabolome, helping to understand how gene regulation influences protein synthesis and enzymes affect certain metabolic pathways. Integrated models may become available, by which not only disease progression could be monitored but also medical treatment success from the very onset or after a short time of application. Even a combinatorial medical approach may become reachable, which could help to predict efficiencies and new targets of either newly discovered pharmaceutical leads or already existing drugs [79].

The main biochemical building blocks of cellular membranes are phospholipids, comprising of a head group and two distinct fatty acids. Choline, ethanolamine, glycerol, and inositol are possible head groups, while fatty acids in the range from C12 to C28 (either unsaturated or saturated with 1–4 double bonds) are building blocks for the fatty acid side chains of each phospholipid. In addition, sphingomyelins, ceramides, and cardiolipins can be found in cellular membranes [80]. Since each phospholipid is made of two fatty acids, a large number of different molecule species can be present in cellular membranes. Nowadays, it becomes clear that the exact composition of the cell membrane, along with the spatial distribution of individual phospholipids, is a key factor not only for cellular transport mechanisms, like proton pumps or other ion channels, but also in cell signaling processes [81]. Therefore, phospholipid patterns could be used, for instance, as biomarkers for the discrimination of different cancer progression stages toward metastasis [82]. Accordingly, specific phospholipid classes have been linked to a variety of different cancer diseases. For instance, increased phosphoinositide levels are characteristic in lung, gastric, prostate, ovarian, and breast cancer [83–86]. At certain stages, tumor cells occasionally start synthesizing a variety of signaling lipids like angiogenesis-promoting prostaglandins [87] or sphingosine-1-phosphate [88], most of which are subsequently secreted into the extracellular matrix. As an example, lysophosphatidic acids have been found increased in serum of ovarian cancer patients [89] and identified as signaling molecules for the regulation of cancer cell proliferation, biochemical resistance to chemotherapy, and radiotherapy-induced apoptosis [90, 91]. Lysophosphatidic acids have also been shown to regulate metalloproteinase activity, a key process during metastasis [92].

Beyond cancer, lipidomics can be used to diagnose a broad variety of other diseases as well and may be the ideal tool to early onset diagnosis even of lifestyle-related diseases like obesity and others [93]. Since not only alterations of phospholipid patterns can be monitored but also fatty acid biosynthesis-related lipid changes, functional signaling molecules along with their biosynthetic precursors can be observed at very early stages where other medical diagnostic tools usually fail. A vibrant example is the downregulation of the stearoyl-CoA desaturase 1 in inflammatory diseases like acute colitis [94]. Higher levels of the saturated stearoyl lysophosphatidylcholine and the unsaturated oleoyl phosphatidylcholine were observed in mice prior to the actual onset of acute colitis symptoms in these animals [95]. If studies

in humans would offer similar results, a novel diagnostic tool for acute colitis may be available in the future that could pave the way for precautionary medicine and thus being invaluable for earliest preonset medical treatment measures.

Lipidomics Approaches and Fields of Application

Currently two different approaches are applied in the lipidomics field. An LC-based approach, which puts a separation step in front of the mass spectral analysis, and a shotgun approach [96]. For the latter, a complex mixture is directly injected into the mass spectrometer and subsequently scanned for specific neutral loss or precursor ions in either negative or positive mode. Specific precursor ion scans and neutral loss scans hereby define different phospholipid head groups [97]. The method is used in combination with different sample dilution and pre-preparation methods, like the addition of LiOH, to analyze all phospholipids from one sample [97]. As an advantage, the analysis time can be reduced to a minimum for each sample.

Multidimensional MS-based quantitative shotgun lipidomics is a valuable tool for lipidomics. The first dimension is the identification of the molecular ion. The precursor ion scan or neutral loss scan identifies the phospholipid class, being phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, sphingomyelin, ceramide, or cardiolipin [97]. From the same spectrum, the molecular ion is accessible. The second dimension subsequently provides an MS/MS experiment of the identified molecular ion, resulting in the identification of the fatty acid constituents of the phospholipid under consideration (Fig. 1). In this way, an exact identification of each phospholipid is possible. Using a set of internal standard compounds for each phospholipid class, a semiquantification of all phospholipids becomes feasible. In that way, more than 2,000 different compounds can be quantified simultaneously from one sample [97]. For instance, the method was applied to identify lipid changes in patients being in the mild cognitive impairment stage of Alzheimer's disease [98]. At present, there exists only one valuable genetic risk factor for late-onset Alzheimer's disease, that is, the ε4 allele of apolipoprotein E [99]. The lipidomics method applied, however, was successful in the identification of additional significant alterations in brain samples of these patients that pertain to the levels of ceramides, sulfatides, and plasmalogens [98].

Eicosanoid level changes have been investigated to diagnose early stages of Lyme arthritis and compare them to late-stage disease patterns [100]. The use of a comprehensive lipidomics approach revealed a complex interaction of different metabolic pathways. Besides the expected regulation of Lyme disease pathology by the enzyme cyclooxygenase, significant downregulation of other metabolic pathways like the lipoxygenase pathway was observed. This study clearly demonstrates that a holistic lipidomics and metabolomics approach is needed to fully understand underlying metabolic profile changes for diseases diagnostics [100]. Only the analysis of as-much-as-possible metabolites guarantees the necessary set of unbiased data needed for the detection of early-onset stages of diseases that may emerge on the background of a complex matrix of dietary intake patterns and the

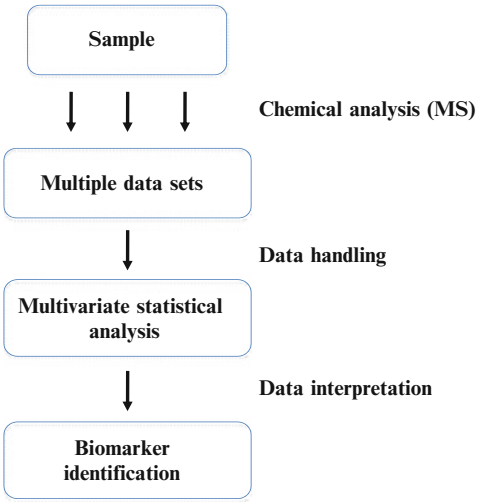


Fig. 1 Principles of multidimensional MS-based quantitative shotgun lipidomics

interfering exposures to environmental chemicals [101]. In first approximation, largest datasets will be required to correlate disease patterns with dietary profiles and toxic chemical influences with the goal to evaluate the best possible treatment of aberrant stages toward healthy profiles. The expression "healthy profile" is not as static as expected in the past; metabolic profiles may change during lifetime with age and with the change of dietary intake patterns or exposures to a variety of environmental factors [102]. Therefore, in-depth scientific assessments of healthy metabolite ranges for a variety of metabolic pathways, which guarantee a stable and healthy life but which may change significantly over lifetime, may be one important focus and a major task of future medical and health-care research. The establishment of such "healthy metabolic profile matrices" has the potential of also being tightly connected to possible high risk groups or more volatile subject groups with certain preconditions, such as asthma or allergies [103]. The primary focus is to identify individuals at risk due to either genetic predisposition or environmental exposures against all kinds of noxa, no matter whether biological, physical, or chemical. Based on this, people can be closely monitored for their individual metabolite patterns to allow for earliest detection of possible detrimental deviations already from the very onset.

Using lipidomics, possible biochemical mechanistic pathways for a variety of diseases may be elucidated in a similar way, and therefore, early onsets of these diseases may become diagnosable in medicine, guaranteeing the earliest treatment interference possible [104]. To this end, an integrated metabolic model is needed, where all available data from a variety of different experiments are being collected and compared to various different metabolic profiles. Multivariate statistics may be implemented together with machine learning networks to evaluate the data and to obtain an individual model-related output reading for each patient. Based on that output, personalized medical treatment strategies together with the reduction of negative toxic environmental factors and/or diet changes may be proposed [105].

Personalized Medicine

The deciphering of the human genome enabled a complete new approach to medicine [106]. All of a sudden, it became possible to tailor medicine to the exact needs of each individual person based on its individual genomic information. Using this approach could ultimately lead to individualized drug doses and prescription regimes, thereby avoiding drug side effects [107]. On the other hand, the available genetic information could also be used to predict and monitor treatment success from the very onset of medical intervention. Tools like genomics, proteomics, metabolomics, and lipidomics could be used to get insights into a person's predisposition to a particular disease. The under- or overexpression of specific genes in correlation with enzyme regulation and the actual metabolomic outcome could guide individual medical treatment. Currently, the field of cancer treatment is probably the most affected by personalized medical approaches [108]. There already exists a variety of different drugs for which patient pretesting is recommended before the actual treatment starts [109, 110]. Moreover, information from lung cancer trials suggests that BRCA1 expression levels could be used to predict drug efficiency and treatment success [111, 112]. Similarly, a genetic germ-line mutation testing regime is proposed to target the right patient subgroup for newly discovered drugs like PARP inhibitors [113].

Personalized medicine uses new analytical techniques to develop improved therapy regimens [114]. It could also lead to a better fine tuning of risk evaluation and treatment benefit for specific subgroups of patients. The definition of different risk groups based on genomics, proteomics, and metabolomics data is probably one of the major promising tasks in future medical treatments [115]. Based on these subgroups, different treatment approaches could become available. However, besides new treatment approaches, also new regulatory testing regimes will become necessary. To fulfill the personalized requirements, drugs will have to undergo a different testing regime to common praxis, including tests for metabolomic changes in target organs or tissues alongside with subgroup testing for metabolomic profile changes in urine and plasma. The acquired data will need an integrated approach to pinpoint ideal population subgroups for newly discovered drugs. Also, new kinds of clinical trials are needed to include a variety of different genetic and metabolomic subgroups in existing testing regimes to highlight ideal treatment doses and patient groups. As a consequence, better statistical tools for data interpretation will be needed, in combination with new approaches in defining the correct confidence intervals and threshold settings for each testing regime and in the development and validation of new biomarkers.

In addition, nutriceuticals may become an integrated part of personalized medicine [116]. Based on metabolic data, dietary intervention could be achieved, and aberrant metabolic profiles could be corrected using a variety of different nutrients. Basic data about the up- and downregulation of metabolic pathways after food intake may give significant indications for the use of a supplementary diet-based approach in health care and possibly also medicinal treatment regimes [117]. Comparing aberrant metabolic patterns with the changes in metabolic profiles may ultimately open up new treatment arrangements based on nutrients or nutriceuticals either alone or in

combination with pharmaceutical drugs. Nutriceuticals are already successfully used as antithrombotic agents. Especially dietary antioxidants like γ-tocopherol decreased the risk of thrombotic events and directly influenced the lipid profile [118]. Also, selenium supplementation showed enhanced levels of prostacyclin I_2 and decreased levels of thromboxane A_2, which indicates the possible therapeutic significance of selenium levels for platelet activation processes [119]. Clinical studies in humans show that coenzyme Q_{10}, given with the diet during cancer therapy, prevents cardiotoxicity from drugs like doxorubicin and daunorubicin [120]. Such studies provide evidence that coexposure to pharmaceuticals and nutrients affords beneficial side effects and can actually be used to regulate adverse drug effects. Here, it seems that a totally new approach in medical treatment becomes conceivable that can be supported and promoted via metabolic pattern profiling and monitoring.

Adding even more on this, an epidemiological study on the benefits of dietary broccoli intake showed for the first time that diets rich in cruciferous vegetables may reduce the risk of prostate cancer by significantly influencing inflammatory signaling pathways [121]. Beyond health care and disease prevention, metabolic modulators may also reveal valuable as integrative components of new treatment strategies. This has been shown, for example, in coronary artery disease patients with ranolazine and trimetazidine [122–124]. Treatment of chronic angina pectoris with either compound reveals inhibitory interferences with specific reactions of the fatty acid oxidation metabolic pathway, which are characteristic for cardiac ischemia patients. In such patients, increased levels of lysolecithins, free arachidonic acid, and acyl carnitines are accompanied by a decrease in the levels of free carnitine [125]. Both drugs were also of value in the clinical treatment of ischemic cardiac disease. The great benefit came from their positive effects without influencing heart rates or blood pressure at the same time. Another compound, dichloroacetate, revealed as inhibitor of pyruvate dehydrogenase activity that enhances carbohydrate oxidation and utilization and diminishes fatty acid oxidation [126]. If metabolomics and lipidomics data would show that fatty acid oxidation is enhanced in other diseases as well, an indication of these pharmaceuticals in combination with other drugs would be conceivable to reduce enhanced fatty acid oxidation without negative side effects occurring from high blood pressure and enhanced heart rate. As last example, the nutriceutical D-ribose enhances ATP synthesis and thus a metabolic pathway which is significantly reduced in patients with coronary artery disease [127].

Human Biomonitoring

Due to the mutual influence of genetic predisposition, lifestyle, diet, and environmental toxin exposure in chronic diseases [128], and especially in cancer development [129], human biomonitoring and an adequately resulting risk assessment from biomarker evaluation have become pivotal in medical research [130]. Generally, biochemicals and chemicals or their metabolites are being analyzed in biological matrices like urine, blood, hairs, saliva, nails, or even tear fluid [131–133].

One of the major tasks of biomonitoring is the comparison between background levels of biomarkers, which can either be derived from literature or from control groups. These background levels are then used in combination with data from actual exposure studies for health risk assessment and the establishment of toxic threshold levels for environmental pollutants and toxicants [134]. Large-scale population studies were conducted by the US Centers for Disease Control and Prevention (CDC) and the US Environmental Protection Agency (US EPA) to evaluate human exposure to environmental chemicals and their fate in the organism including bioaccumulation [135, 136]. Currently, there exists a European Union (EU) sponsored coordination program (COPHES: Consortium to Perform Human Biomonitoring on a European Scale) to develop a coherent approach of human biomonitoring in Europe as requested by the EU Environment and Health Action Plan (see: http://www.eu-hbm.info/cophes). An extremely valuable approach in biomonitoring is the collection and evaluation of reference material as realized by the German National Environmental Specimen Bank (ESB; see http://www.umweltprobenbank.de/en/documents). The ESB was established under the supervision of the German Federal Ministry for the Environment, Nature Conservation and Nuclear Safety and collects environmental and human specimens (blood, urine, hairs, *etc*.) from the German population at regular intervals. Data are anonymous and stored together with the collection of a standardized questionnaire to provide answers on living circumstances, exposure levels, and nutrition habits. Data were used, for instance, to investigate and review heavy metal accumulation and changes in aquatic environments over the past 20 years [137] and to evaluate the levels of perfluorinated compounds in human plasma samples collected from contaminated sites over the past decades [138]. Especially human biomonitoring data from children are needed due to specific metabolic differences between children and adults, resulting not only in different metabolic profiles but also in different turnover rates of pharmaceuticals and nutrients. In Germany, the German Environmental Survey for Children (GerES IV) gathers important information about possible adverse health effects in the young resulting from exposures to environmental pollutants, tobacco smoke, and other noxa [139, 140].

Human biomonitoring data provide valuable information on which chemicals are actually being incorporated into humans and which among them can bioaccumulate in human tissues [141]. Further, concentration levels of specific chemicals can be monitored time-dependently and evaluated in light of exposure hot spots or worldwide usage of these compounds. Additionally, information about the distribution among the general population is obtainable. To this end, specimen collections for urine and blood samples are indeed one prerequisite in human biomonitoring [142, 143]. A variety of different studies were already conducted worldwide. Nowadays, additional long-term human biomonitoring studies are anticipated. The DONALD (Dortmund Nutritional and Anthropometric Longitudinally Designed) study investigates newborns from birth to adulthood in the German town Dortmund [144]. Every year, some 40 newborns were recruited for the study, 1,100 examined infants so far. Data evaluated comprise diet, growth, and metabolic parameters like urine specimens and medical examinations. Based on nutritional, environmental, and medical data, an overall assessment of exposure to environmental chemicals will become available. Genetic investigations allow risk prediction of a variety of diseases like cancer or

coronary heart disease [145]. Biomonitoring studies like the DONALD study allow risk prediction models to be evaluated later on with real life data. Therefore, better risk prediction models will become available after data interpretation. Similar studies may help to better understand baseline levels of metabolites in single individuals, and their healthy threshold levels may become accessible, simply by investigating time-dependent metabolite profiles in individuals who eventually develop conditions like cancer or coronary heart disease later in life. In that way, early stages of aberrant metabolic and nutritional profiles may become accessible, thus paving the road toward personalized medicine in the future.

Another tool in human biomonitoring is the chemical analysis of human breast milk to evaluate possible environmental health risk for infants [146]. Especially newborns are receptible and vulnerable to a variety of hazardous compounds like neurotoxins or developmental regulators [147, 148]. A study about polybrominated diphenyl ethers in human breast milk showed the possible interaction between maternal characteristics and environmental factors like dietary intake and living conditions [146]. Human milk may actually be very well suited to monitor the health burden resulting from infant exposure to chemicals and to evaluate geographical trends for differences in environmental exposures. Additionally, human biomonitoring data could also be used for risk assessment of environmental toxins [149]. A huge amount of biokinetic parameters have been established in animal- or cell- and tissue-based toxicology studies [150]. Simplified models are needed to study the relationship between exposure to a given chemical and its bioaccumulation in specific body tissues or organs and fluids, which ideally are based on animal toxicological data or even better on human cell-based bioassays. Physiologically based toxicokinetic (PBTK) models are already successfully used to determine interindividual variability in metabolism and biokinetics of drugs and environmental chemicals [151]. Combining these data with biomonitoring studies, starting in newborns and later on periodically conducted until adulthood, may finally lead to disease prediction models covering the entire life span and smooth the way to sophisticated preventive medicine.

Additional subpopulation monitoring becomes increasingly important [152]. To decide whether or not certain concentrations of environmental chemicals may have a negative health impact on a specific individual, population subgrouping is a very useful tool. The goal is to set up a certain number of population subgroups, each of which with similar genetic or metabolic backgrounds, to afford better assumptions on possible health impacts of drugs and environmental exposures. For each subgroup, specific threshold levels for the same drug or environmental chemical are thinkable, thus guaranteeing much better health safety levels due to individualization.

Summary

Future medicine may look completely different from the health system currently in place. New approaches in disease prediction and early diagnosis, in combination with true personalized medicines relying more on so-called niche busters than general blockbusters, may be the way forward toward a more efficient and

cost-effective worldwide health system. Personalized datasets from "cradle to grave" based on genetic, proteomic, and metabolomic monitoring could be used to generate new insights into profile changes caused by diseases, obesity, malnutrition, insomnia, stress, and environmental pollutants and its mutual interactions. Worldwide human biomonitoring of large population groups is necessary to evaluate biomarkers and metabolic profiles and to generate the basic datasets for the evaluation of profile changes and population subgroup thresholds. Information from beneficial nutrients and natural products as well as pharmaceuticals and their interactions guarantee a better understanding in personalized treatment arrangements and therapeutic monitoring and may bridge the way for a new proactive health-care system. This may not only consist of pharmaceuticals but also include daily diet and the use of natural herbal products and remedies. The mutual interaction of health, diet, and physiological steady state needs a much deeper scientific insight to understand the actual outcome of the regulatory measures which need to be applied in public health prevention and medical treatment.

The combination of human biomonitoring and genomics, epigenetics, proteomics, and metabolomics data sets may not only path the way to more cost-effective medical treatment due to the detection of diseases in the very early stages but may also improve the quality of life and finally result in a new and improved health service for mankind.

References

1. McEwen BS, Stellar E (1993) Stress and the individual. Mechanisms leading to disease. Arch Intern Med 153:2093–2101
2. Shaw KJ, Birch C, Hughes EM, Jakes AD, Greeenman J, Haswell ST (2011) Microsystems for personalized biomolecular diagnostics. Eng Life Sci 2:121–132
3. Abelev GI, Perova SD, Khramkova NI, Postnikova ZA, Irlin IS (1963) Production of embryonal α-globulin by transplantable mouse hepatomas. Transplantation 1:174–180
4. Wide L, Gemzell CA (1960) An immunological pregnancy test. Acta Endocrinol 35: 261–267
5. Yalow RS, Berson SA (1959) Assay of plasma insulin in human subjects by immunological methods. Nature 184(Suppl 21):1648–1649
6. Thomson DM, Krupey J, Freedman SO, Gold P (1969) The radioimmunoassay of circulating carcinoembryonic antigen off the human digestive system. Proc Natl Acad Sci USA 64: 161–167
7. Homburger HA, Miller SA, Jacob GL (1980) Radioimmunoassay of creatine kinase B-isoenzymes in serum of patients with azotemia, obstructive uropathy, or carcinoma of the prostate or bladder. Clin Chem 26:1821–1824
8. Eriksson B, Arnberg H, Oberg K, Hellman U, Lundqvist G, Wernstedt C, Wilander E (1990) A polyclonal antiserum against chromogranin A and B—A new sensitive marker for neuroendocrine tumours. Acta Endocrinol 122:145–155
9. Grebenchtchikov N, Geurts-Moespot AJ, Kroot JJ, den Heijer M, Tjalsma H, Swinkels DW, Sweep FG (2009) High-sensitive radioimmunoassay for human serum hepcidin. Br J Haematol 146:317–325
10. Mirick DK, Davis S (2008) Melatonin as a biomarker of circadian dysregulation. Cancer Epidemiol Biomarkers Prev 17:3306–3313

11. Lucato RV Jr, Abdulkader RC, Barbaro KC, Mendes GE, Castro I, Baptista MA, Cury PM, Malheiros DM, Schor N, Yu L, Burdmann EA (2011) *Loxosceles* gaucho venom-induced acute kidney injury—*In vivo* and *in vitro* studies. PloS Negl Trop Dis 5:e1182
12. LaDue JS, Wróblewski F, Karmen A (1954) Serum glutamic oxaloacetic transaminase activity in human acute transmural myocardial infarction. Science 120:497–499
13. Nissen NI, Ranlov P, Weis-Fogh J (1965) Evaluation of four different serum enzymes in the diagnosis of acute myocardial infarction. Br Heart J 27:520–526
14. Dreyfus JC, Schapira G, Rasnais J, Scebat L (1960) Serum creatine kinase in the diagnosis of myocardial infarct. (La creatine-kinase sérique dans le diagnostic de l'infarctus myocardique.). Rev Fr Etud Clin Biol 5:386–387
15. Burger R, Richterich R, Aebi H (1964) The heterogenicity of creatine kinase. Biochem Z 339:305–314
16. Konttinen A, Somer H (1972) Determination of serum creatine kinase isoenzymes in myocardial infarction. Am J Cardiol 29:817–820
17. Roberts R, Henry PD, Sobel BE (1975) An improved basis for enzymatic estimation of infarct size. Circulation 52:743–754
18. Katus HA, Remppis A, Looser S, Hallermeier K, Scheffold T, Kübler W (1989) Enzyme linked immuno assay of cardiac troponin T for the detection of acute myocardial infarction in patients. J Mol Cell Cardiol 21:1349–1353
19. Wang TJ (2011) Assessing the role of circulating, genetic, and imaging biomarkers in cardiovascular risk prediction. Circulation 123:551–565
20. Fletcher JS, Lockyer NP, Vaidyanathan S, Vickerman JC (2007) TOF-SIMS 3D biomolecular imaging of *Xenopus laevis* oocytes using buckminsterfullerene (C_{60}) primary ions. Anal Chem 79:2199–2206
21. Jungnickel H, Jones EA, Lockyer NP, Oliver SG, Stephens GM, Vickerman JC (2005) Application of TOF-SIMS with chemometrics to discriminate between four different yeast strains from the species *Candida glabrata* and *Saccharomyces cerevisiae*. Anal Chem 77:1740–1745
22. Kannel WB, McGee D, Gordon T (1976) A general cardiovascular risk profile: the Framingham Study. Am J Cardiol 38:46–51
23. D'Agostino RB (2006) Risk prediction and finding new independent prognostic factors. J Hypertens 24:643–645
24. Zhou XH, Obuchowski NA, McClish DK (2002) Statistical methods in diagnostic medicine. Wiley, New York, NY
25. Honzík P, Krivan L, Lokaj P, Lábrová R, Nováková Z, Fiser B, Honzíková N (2010) Logit and fuzzy models in data analysis: estimation of risk in cardiac patients. Physiol Res 59(Suppl 1):S89–S96
26. Steyerberg EW, Pencina MJ, Lingsma HF, Kattan MW, Vickers AJ, Van Calster B (2012) Assessing the incremental value of diagnostic and prognostic markers: a review and illustration. Eur J Clin Invest 42:216–228
27. Zhu H, Zhang Q, Wu DL, Fu S (2008) The value of serum PSA subgroups and biopsy Gleason score in the prediction of pathologic stage of prostate cancer. Tumor 28:613–616
28. Lê Cao KA, González I, Déjean S (2009) integrOmics: an R package to unravel relationships between two omics datasets. Bioinformatics 25:2855–2856
29. Beger RD, Colatsky T (2012) Metabolomics data and the biomarker qualification process. Metabolomics 8:2–7
30. Danielsson R, Allard E, Sjöberg PJR, Bergquist J (2011) Exploring liquid chromatography-mass spectrometry fingerprints of urine samples from patients with prostate or urinary bladder cancer. Chemom Intellig Lab Syst 108:33–48
31. Blamek S, Larysz D, Ficek K, Sokól M, Miszczyk L, Tarnaweski R (2010) MR spectroscopic evaluation of brain tissue damage after treatment for pediatric brain tumors. Acta Neurochir Suppl 106:183–186
32. Lee K, Hwang D, Yarmush ML, Stephanopoulos GN (2002) Metabolic profiling: definition and use in physiological state classification. Engin Med Biol 3:2213–2214

33. Wang JH, Byun J, Pennathur S (2010) Analytical approaches to metabolomics and applications to systems biology. Semin Nephrol 30:500–511
34. Smilde AK, van der Werf MJ, Bijlsma S, van der Werff-van der Vat BJC, Jellema RH (2005) Fusion of mass spectrometry-based metabolomics data. Anal Chem 77:6729–6736
35. Moco S, Bino RJ, De Vos RCH, Vervoort J (2007) Metabolomics technologies and metabolite identification. Trends Anal Chem 26:855–866
36. Weinstein JN (2006) Spotlight on molecular profiling: "Integromic" analysis of the NCI-60 cancer cell lines. Mol Cancer Ther 5:2601–2605
37. Tiziani S, Lopes V, Günther UL (2009) Early stage diagnosis of oral cancer using ^{1}H NMR-based metabolomics. Neoplasia 11:269–276
38. Shi H, Paolucci U, Vigneau-Callahan KE, Milbury PE, Matson WR, Kristal BS (2004) Development of biomarkers based on diet-dependent metabolic serotypes: practical issues in development of expert system-based classification models in metabolomic studies. OMICS 8:197–208
39. Vinayavekhin N, Homan EA, Saghatelian A (2010) Exploring disease through metabolomics. ACS Chem Biol 5:91–103
40. Lu W, Bennett BD, Rabinowitz JD (2008) Analytical strategies for LC-MS-based targeted metabolomics. J Chromatogr B Analyt Technol Biomed Life Sci 871:236–242
41. Wei R, Li G, Seymour AB (2010) High-throughput and multiplexed LC/MS/MRM method for targeted metabolomics. Anal Chem 82:5527–5533
42. Zhang GF, Sadhukhan S, Tochtrop GP, Brunengraber H (2011) Metabolomics, pathway regulation, and pathway discovery. J Biol Chem 286:23631–23635
43. Gonzalez E, van Liempd S, Conde-Vancells J, Gutierrez-de Juan V, Perez-Cormenzana M, Mayo R, Berisa A, Alonso C, Marquez CA, Barr J, Falcon-Perez JM (2011) Serum UPLC-MS/MS metabolic profiling in an experimental model for acute-liver injury reveals potential biomarkers for hepatotoxicity. Metabolomics. doi:10.1007/s11306-011-0329-9
44. Zhang L, Jia X, Peng X, Ou Q, Zhang Z, Qiu C, Yao Y, Shen F, Yang H, Ma F, Wang J, Yuan Z (2010) Development and validation of a liquid chromatography-mass spectrometry metabolomic platform in human plasma of liver failure caused by hepatitis B virus. Acta Biochim Biophys Sin 42:688–698
45. Le Gall G, Colquhoun IJ, Defernez M (2004) Metabolite profiling using ^{1}H NMR spectroscopy for quality assessment of green tea, *Camellia sinensis* (L.). J Agric Food Chem 52:692–700
46. Lan K, Zhang Y, Yang J, Xu L (2010) Simple quality assessment approach for herbal extracts using high performance liquid chromatography—UV based metabolomics platform. J Chromatogr A 1217:1414–1418
47. Tiran D (2012) Ginger to reduce nausea and vomiting during pregnancy: evidence of effectiveness is not the same as proof of safety. Complement Ther Clin Pract 18:22–25
48. Weber W, Luzi S, Karlsson M, Fussenegger M (2009) A novel hybrid dual-channel catalytic-biological sensor system for assessment of fruit quality. J Biotechnol 139:314–317
49. Tsouti V, Boutopoulos C, Zergioti I, Chatzandroulis S (2011) Capacitive microsystems for biological sensing. Biosens Bioelectron 27:1–11
50. Ruiz-Aracama A, Peijnenburg A, Kleinjans J, Jennen D, van Delft J, Hellfrisch C, Lommen A (2011) An untargeted multi-technique metabolomics approach to studying intracellular metabolites of HepG2 cells exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. BMC Genomics 12:251–270
51. Kusano M, Fukushima A, Arita M, Jonsson P, Moritz T, Kobayashi M, Hayashi N, Tohge T, Saito K (2007) Unbiased characterization of genotype-dependent metabolic regulations by metabolomic approach in *Arabidopsis thaliana*. BMC Syst Biol 1:53–69
52. Rijk JC, Lommen A, Essers ML, Groot MJ, Van Hende JM, Doeswijk TG, Nielen MW (2009) Metabolomics approach to anabolic steroid urine profiling of bovines treated with prohormones. Anal Chem 81:6879–6888

53. Michopoulos F, Theodoridis G, Smith CJ, Wilson ID (2010) Metabolite profiles from dried biofluid spots for metabonomic studies using UPLC combined with oaToF-MS. J Proteome Res 9:3328–3334
54. Sana TR, Roark JC, Li X, Waddell K, Fischer SM (2008) Molecular formula and METLIN Personal Metabolite Database matching applied to the identification of compounds generated by LC/TOF-MS. J Biomol Tech 19:258–266
55. Melamud E, Vastag L, Rabinowitz JD (2010) Metabolomic analysis and visualization engine for LC-MS data. Anal Chem 82:9818–9826
56. Ahn SY, Jamshidi N, Mo ML, Wu W, Eraly SA, Dnyanmote A, Bush KT, Gallegos TF, Sweet DH, Palsson BØ, Nigam SK (2011) Linkage of organic anion transporter-1 to metabolic pathways through integrated "omics"-driven network and functional analysis. J Biol Chem 286:31522–31531
57. Marhuenda-Egea FC, Gonsálvez-Álvarez R, Martínez-Sabater E, Lledó B, Ten J, Bernabeu R (2011) Improving human embryos selection in IVF: non-invasive metabolomic and chemometric approach. Metabolomics 7:247–256
58. Thomas C, Moridani M (2011) Cancer vaccines: personalizing health interventions. Curr Pharmacogen Personal Med 9:208–228
59. Tweed JA, Gu Z, Xu H, Zhang G, Nouri P, Li M, Steenwyk R (2010) Automated sample preparation for regulated bioanalysis: an integrated multiple assay extraction platform using robotic liquid handling. Bioanalysis 2:1023–1040
60. Zhang J, Wei S, Ayres DW, Smith HT, Tse FL (2011) An automation-assisted generic approach for biological sample preparation and LC-MS/MS method validation. Bioanalysis 3:1975–1986
61. Tattersall P, Kubin M, Hussain A (2010) Monitoring GMP compliance in an analytical R&D laboratory by use of a peer-led program. Am Pharm Rev 13:114–119
62. Schlegel J (2000) Flexible, parameter-controlled nanoliter pipetting on a Tecan xyz platform. J Assoc Lab Autom 5:72–74
63. Jokerst JV, Chou J, Camp JP, Wong J, Lennart A, Pollard AA, Floriano PN, Christodoulides N, Simmons GW, Zhou Y, Ali MF, McDevitt JT (2011) Location of biomarkers and reagents within agarose beads of a programmable bio-nano-chip. Small 7:613–624
64. Pagaduan JV, Yang W, Woolley AT (2011) Optimization of monolithic columns for microfluidic devices. In: Proceedings of the conference on micro- and nanotechnology sensors, systems, and applications, Orlando, FL, USA. Proc SPIE Int Soc Optical Eng 8031:artno-83311V
65. Xu Z, Koshimidzu E, Hirokawa T (2009) Electrokinetic sample injection for high-sensitivity CZE (part 2): Improving the quantitative repeatability and application of electrokinetic supercharging-CZE to the detection of atmospheric electrolytes. Electrophoresis 30:3534–3539
66. Akira K, Mitome H, Imachi M, Shida Y, Miyaoka H, Hashimoto T (2010) LC-NMR identification of a novel taurine-related metabolite observed in ^{1}H NMR-based metabonomics of genetically hypertensive rats. J Pharm Biomed Anal 51:1091–1096
67. Sun J, Schnackenberg LK, Pence L, Bhattacharyya S, Doerge DR, Bowyer JF, Beger RD (2010) Metabolomic analysis of urine from rats chronically dosed with acrylamide using NMR and LC/MS. Metabolomics 6:550–563
68. Capuano E, Fogliano V (2011) Acrylamide and 5-hydroxymethylfurfural (HMF): a review on metabolism, toxicity, occurrence in food an mitigation strategies. LWT Food Sci Technol 44:793–810
69. Hasegawa M, Ide M, Kuwamura M, Yamate J, Takenaka S (2010) Metabolic fingerprinting in toxicological assessment using FT-ICR MS. J Toxicol Pathol 23:67–74
70. Pan X, Wilson M, Mirbahai L, McConville C, Arvanitis TN, Griffin JL, Kauppinen RA, Peet AC (2011) *In vitro* metabonomic study detects increases in UDP-GlcNAc and UDP-GalNAc, as early phase markers of cisplatin treatment response in brain tumor cells. J Proteome Res 10:3493–3500
71. Lawrence DM (2010) Healthcare for elders in 2050. Generations 34:82–85

72. Choi MY, Choi W, Park JH, Lim J, Kwon SW (2010) Determination of coffee origins by integrated metabolomic approach of combining multiple analytical data. Food Chem 121: 1260–1268
73. Frühwirth R, Mani DR, Pyne S (2011) Clustering with position-specific constraints on variance: applying redescending M-estimators to label-free LC-MS data analysis. BMC Bioinformatics 12:358–370
74. Xia J, Wishart DS (2010) MSEA: a web-based tool to identify biologically meaningful patterns in quantitative metabolomic data. Nucleic Acids Res 38:W71–W77
75. Guichardant M, Chen P, Liu M, Calzada C, Colas R, Véricel E, Lagarde M (2011) Functional lipidomics of oxidized products from polyunsaturated fatty acids. Chem Phys Lipids 164: 544–548
76. Bird SS, Marur VR, Sniatynski MJ, Greenberg HK, Kristal BS (2011) Serum lipidomics profiling using LC-MS and high-energy collisional dissociation fragmentation: focus on triglyceride detection and characterization. Anal Chem 83:6648–6657
77. Fernandis AZ, Wenk MR (2009) Lipid-based biomarkers for cancer. J Chromatogr B Analyt Technol Biomed Life Sci 877:2830–2835
78. Salem G (2011) Integrated biology: multiple omics. Eur Biopharm Rev 1:46–51
79. Jung HR, Sylvänne T, Koistinen KM, Tarasov K, Kauhanen D, Ekroos K (2011) High throughput quantitative molecular lipidomics. Biochim Biophys Acta 1811:925–934
80. Berg JM, Tymoczko JL, Stryer L (2002) Biochemistry. Chap 12: Lipids and cell membranes, 5th edn. WH Freeman, New York, NY
81. Valluru R, Van den Ende W (2011) Myo-inositol and beyond—emerging networks under stress. Plant Sci 181:387–400
82. Hilvo M, Denkert C, Lehtinen L, Müller B, Brockmöller S, Seppänen-Laakso T, Budczies J, Bucher E, Yetukuri L, Castillo S, Berg E, Sysi-Aho M, Griffin JL, Fiehn O, Loibl S, Richter-Ehrenstein C, Radke C, Hyötyläinen T, Kallioniemi O, Iljin K, Oresic M (2011) Novel theranostic opportunities offered by characterization of altered membrane lipid metabolism in breast cancer progression. Cancer Res 71:3236–3245
83. Ringel MD, Hayre N, Saito B, Saunier B, Schuppert F, Burch H, Bernet V, Burman KD, Kohn LD, Saji M (2001) Overexpression and overactivation of Akt in thyroid carcinoma. Cancer Res 61:6105–6111
84. Yan W, Fu Y, Tian D, Liao J, Liu M, Wang B, Xia L, Zhu Q, Luo M (2009) PI3 kinase/Akt signaling mediates epithelial-mesenchymal transition in hypoxic hepatocellular carcinoma cells. Biochem Biophys Res Commun 382:631–636
85. Altomare DA, Wang HQ, Skele KL, De RA, Klein-Szanto AJ, Godwin AK, Testa JR (2004) AKT and mTOR phosphorylation is frequently detected in ovarian cancer and can be targeted to disrupt ovarian tumor cell growth. Oncogene 23:5853–5857
86. Lia Y, Grobholz R, Abel U, Trojan L, Michel MS, Angel P, Mayer D (2003) Increase of AKT/PKB expression correlates with gleason pattern in human prostate cancer. Int J Cancer 107:676–680
87. Wang MT, Honn KV, Nie D (2007) Cyclooxygenases, prostanoids, and tumor progression. Cancer Metastasis Rev 26:525–534
88. Sarkar S, Maceyka M, Hait NC, Paugh SW, Sankala H, Milstien S, Spiegel S (2005) Sphingosine kinase 1 is required for migration, proliferation and survival of MCF-7 human breast cancer cells. FEBS Lett 579:5313–5317
89. Sutphen R, Xu Y, Wilbanks GD, Fiorica J, Grendys EC Jr, LaPolla JP, Arango H, Hoffman MS, Martino M, Wakeley K, Griffin D, Blanco RW, Cantor AB, Xiao YJ, Krischer JP (2004) Lysophospholipids are potential biomarkers of ovarian cancer. Cancer Epidemiol Biomarkers Prev 13:1185–1191
90. Meleh M, Pozlep B, Mlakar A, Meden-Vrtovec H, Zupancic-Kralj L (2007) Determination of serum lysophosphatidic acid as a potential biomarker for ovarian cancer. J Chromatogr B Analyt Technol Biomed Life Sci 858:287–291

91. Mills GB, Eder A, Fang X, Hasegawa Y, Mao M, Lu Y, Tanyi J, Tabassam FH, Wiener J, Lapushin R, Yu S, Parrott JA, Compton T, Tribley W, Fishman D, Stack MS, Gaudette D, Jaffe R, Furui T, Aoki J, Erickson JR (2002) Critical role of lysophospholipids in the pathophysiology, diagnosis, and management of ovarian cancer. Cancer Treat Res 107:259–283
92. Gschwind A, Prenzel N, Ullrich A (2002) Lysophosphatidic acid-induced squamous cell carcinoma cell proliferation and motility involves epidermal growth factor receptor signal transactivation. Cancer Res 62:6329–6336
93. Meikle PJ, Christopher MJ (2011) Lipidomics is providing new insight into the metabolic syndrome and its sequelae. Curr Opin Lipidol 22:210–215
94. Brown JM, Rudel LL (2010) Stearoyl-coenzyme A desaturase 1 inhibition and the metabolic syndrome: considerations for future drug discovery. Curr Opin Lipidol 21:192–197
95. Chen C, Shah YM, Morimura K, Krausz KW, Miyazaki M, Richardson TA, Morgan ET, Ntambi JM, Idle JR, Gonzalez FJ (2008) Metabolomics reveals that hepatic stearoyl-CoA desaturase 1 downregulation exacerbates inflammation and acute colitis. Cell Metab 7: 135–147
96. Guo X, Lankmayr E (2010) Multidimensional approaches in LC and MS for phospholipid bioanalysis. Bioanalysis 2:1109–1123
97. Han X, Yang K, Gross RW (2008) Microfluidics-based electrospray ionization enhances the intrasource separation of lipid classes and extends identification of individual molecular species through multi-dimensional mass spectrometry: development of an automated high-throughput platform for shotgun lipidomics. Rapid Commun Mass Spectrom 22:2115–2124
98. Han X (2010) Multi-dimensional mass spectrometry-based shotgun lipidomics and altered lipids at the mild cognitive impairment stage of Alzheimer's disease. Biochim Biophys Acta 1801:774–783
99. Strittmatter WJ, Roses AD (1996) Apolipoprotein E and Alzheimer's disease. Annu Rev Neurosci 19:53–77
100. Blaho VA, Buczynski MW, Brown CR, Dennis EA (2009) Lipidomic analysis of dynamic eicosanoid responses during the induction and resolution of Lyme arthritis. J Biol Chem 284:21599–21612
101. Bujak R, Daghir E, Rybka J, Koslinski P, Markuszewski MJ (2011) Metabolomics in urogenital cancer. Bioanalysis 3:913–923
102. Tyagi S, Raghvendra S, Singh U, Kalra T, Munjal K (2010) Applications of metabolomics—a systematic study of the unique chemical fingerprints: an overview. Int J Pharmaceut Sci Rev Res 3:83–86
103. Saude EJ, Skappak CD, Regush S, Cook K, Ben-Zvi A, Becker A, Mogbel R, Sykes BD, Rowe BH, Adamko DJ (2011) Metabolomic profiling of asthma: diagnostic utility of urine nuclear magnetic resonance spectroscopy. J Allergy Clin Immunol 127:757–764
104. Yang J, Dong H, Hammock BD (2011) Profiling the regulatory lipids: another systemic way to unveil the biological mystery. Curr Opin Lipidol 22:197–203
105. Pryor DI, Solomon B, Porceddu SV (2011) The emerging era of personalized therapy in squamous cell carcinoma of the head and neck. Asia Pac J Clin Oncol 7:236–251
106. Pirmohamed M (2011) Pharmacogenetics: past, present and future. Drug Discov Today 16: 852–861
107. Roedder S, Vitalone M, Khatri P, Sarwal MM (2011) Biomarkers in solid organ transplantation: establishing personalized transplantation medicine. Genome Med 3:37–49
108. Thomas C, Moridani M (2011) Cancer vaccines: personalizing health interventions. Curr Pharmacogenom Personal Med 9:208–228
109. Schaeffeler E, Fischer C, Brockmeier D, Wernet D, Moerike K, Eichelbaum M, Zanger UM, Schwab M (2004) Comprehensive analysis of thiopurine *S*-methyltransferase phenotype-genotype correlation in a large population of German-Caucasians and identification of novel TPMT variants. Pharmacogenetics 14:407–417
110. Hoskins JM, Goldberg RM, Qu P, Ibrahim JG, McLeod HL (2007) UGT1A1*28 genotype and irinotecan-induced neutropenia: dose matters. J Natl Cancer Inst 99:1290–1295

111. Taron M, Rosell R, Felip E, Mendez P, Souglakos J, Ronco MS, Queralt C, Majo J, Sanchez JM, Sanchez JJ, Maestre J (2004) BRCA1 mRNA expression levels as an indicator of chemoresistance in lung cancer. Hum Mol Genet 50:2443–2449
112. Watchers FM, Wong LS, Timens W, Kampinga HH, Groen HJ (2005) ERCC1, hRad51, and BRCA1 protein expression in relation to tumour response and survival of stage III/IV NSCLC patients treated with chemotherapy. Lung Cancer 50:211–219
113. Loveday C, Turnbull C, Ramsay E, Hughes D, Ruark E, Frankum JR, Bowden G, Kalmyrzaev B, Warren-Perry M, Snape K, Adlard JW, Barwell J, Berg J, Brady AF, Brewer C, Brice G, Chapman C, Cook J, Davidson R, Donaldson A, Douglas F, Greenhalgh L, Henderson A, Izatt L, Kumar A, Lalloo F, Miedzybrodzka Z, Morrison PJ, Paterson J, Porteous M, Rogers MT, Shanley S, Walker L, Eccles D, Evans DG, Renwick A, Seal S, Lord CJ, Ashworth A, Reis-Filho JS, Antoniou AC, Rahman N (2011) Germline mutations in RAD51D confer susceptibility to ovarian cancer. Nat Genet 43:879–882
114. Offit K (2011) Personalized medicine: new genomics, old lessons. Hum Genet 130:3–14
115. Özgüç M (2011) Genetic testing: predictive value of genotyping for diagnosis and management of disease. EPMA J 2:173–179
116. Chakrabarti S, Freedman JE (2010) Review: nutriceuticals as antithrombotic agents. Cardiovasc Ther 28:227–235
117. O'Sullivan A, Gibney MJ, Brennan L (2011) Dietary intake patterns are reflected in metabolomic profiles: potential role in dietary assessment studies. Am J Clin Nutr 93: 314–321
118. Singh I, Turner AH, Sinclair AJ, Li D, Hawley JA (2007) Effects of γ-tocopherol supplementation on thrombotic risk factors. Asia Pac J Clin Nutr 16:422–428
119. Abdulah R, Katsuya Y, Kobayashi K, Nakazawa M, Nara M, Murakami M, Koyama H (2007) Effect of sodium selenite supplementation on the levels of prostacyclin I_2 and thromboxane A_2 in human. Thromb Res 119:305–310
120. Conklin KA (2005) Coenzyme q10 for prevention of anthracycline-induced cardiotoxicity. Integr Cancer Ther 4:110–130
121. Traka M, Gasper AV, Melchini A, Bacon JR, Needs PW, Frost V, Chantry A, Jones AM, Ortori CA, Barrett DA, Ball RY, Mills RD, Mithen RF (2008) Broccoli consumption interacts with GSTM1 to perturb oncogenic signalling pathways in the prostate. PLoS One 3:e2568
122. McCormack JG, Staneley WC, Wolff AA (1998) Ranolazine: a novel metabolic modulator for the treatment of angina. Gen Pharmacol 30:639–645
123. Rupp H, Zarain-Herzberg A, Maisch B (2002) The use of partial fatty acid oxidation inhibitors for metabolic therapy of angina pectoris and heart failure. Herz 27:621–636
124. Bhandari B, Subramanian L (2007) Ranolazine, a partial fatty acid oxidation inhibitor, its potential benefit in angina and other cardiovascular disorders. Recent Pat Cardiovasc Drug Discov 2:35–39
125. van der Vusse GJ, Glatz JF, Stam HC, Reneman RS (1992) Fatty acid homeostasis in the normoxic and ischemic heart. Physiol Rev 72:881–940
126. Michelakis ED, Sutendra G, Dromparis P, Webster L, Haromy A, Niven E, Maguire C, Gammer TL, Mackey JR, Fulton D, Abdulkarim B, McMurtry MS, Petruk KC (2010) Metabolic modulation of glioblastoma with dichloroacetate. Sci Transl Med 2:31ra34
127. Perkowski DJ, Wagner S, Schneider JR, St Cyr JA (2011) A targeted metabolic protocol with D-ribose for off-pump coronary artery bypass procedures: a retrospective analysis. Ther Adv Cardiovasc Dis 5:185–192
128. Marcer G, Saia B (1989) Environment and the immune system. G Ital Med Lav 11:27–32
129. Bencko V, Rames J, Ondrusova M, Plesko I, Jurickova L, Trnovec T (2009) Human exposure to polyhalogenated hydrocarbons and incidence of selected malignancies—Central European experience. Neoplasma 56:353–357
130. Stiborova M, Rupertova M, Hodek P, Frei E, Schmeiser HH (2004) Monitoring of DNA adducts in humans and ^{32}P-postlabelling methods: a review. Collect Czech Chem Commun 69:476–498

131. Caporossi L, Santoro A, Papaleo B (2010) Saliva as an analytical matrix: state of the art and application for biomonitoring. Biomarkers 15:475–487
132. Chu M, Shirai T, Takahashi D, Arakawa T, Kudo H, Sano K, Sawada S, Yano K, Iwasaki Y, Akiyoshi K, Mochizuki M, Mitsubayashi K (2011) Biomedical soft contact-lens sensor for *in situ* ocular biomonitoring of tear contents. Biomed Microdevices 13:603–611
133. Genuis SJ, Birkholz D, Rodushkin I, Beesoon S (2011) Blood, urine, and sweat (BUS) study: monitoring and elimination of bioaccumulated toxic elements. Arch Environ Contam Toxicol 61:344–357
134. Sobus JR, Tan YM, Pleil JD, Sheldon LS (2011) A biomonitoring framework to support exposure and risk assessments. Sci Total Environ 409:4875–4884
135. Srogi K (2007) Monitoring of environmental exposure to polycyclic aromatic hydrocarbons: a review. Environ Chem Lett 5:169–195
136. Axelrad DA, Goodman S, Woodruff TJ (2009) PCB body burdens in US women of childbearing age 2001-2002: an evaluation of alternate summary metrics of NHANES data. Environ Res 109:368–378
137. Rüdel H, Fliedner A, Kösters J, Schröter-Kermani C (2010) Twenty years of elemental analysis of marine biota within the German Environmental Specimen Bank—a thorough look at the data. Environ Sci Pollut Res Int 17:1025–1034
138. Wilhelm M, Hölzer J, Dobler L, Rauchfuss K, Midasch O, Kraft M, Angerer J, Wiesmüller G (2009) Preliminary observations on perfluorinated compounds in plasma samples (1977-2004) of young German adults from an area with perfluorooctanoate-contaminated drinking water. Int J Hyg 212:142–145
139. Kolossa-Gehring M, Becker K, Conrad A, Lüdecke A, Riedel S, Seiwert M, Schulz C, Szewzyk R (2007) German Environmental Survey IV for Children (GerES IV)—first results. Int J Hyg Environ Health 210:535–540
140. Conrad A, Schulz C, Seiwert M, Becker K, Ullrich D, Kolossa-Gehring M (2010) German Environmental Survey IV: children's exposure to environmental tobacco smoke. Toxicol Lett 192:79–83
141. Turesky RJ, Le Marchand L (2011) Metabolism and biomarkers of heterocyclic aromatic amines in molecular epidemiology studies: lessons learned from aromatic amines. Chem Res Toxicol 24:1169–1214
142. Wiesmüller GA, Eckard R, Dobler L, Günsel A, Oganowski M, Schröter-Kermani C, Schlüter C, Gies A, Kemper FH (2007) The Environmental Specimen Bank for Human Tissues as part of the German Environmental Specimen Bank. Int J Hyg Environ Health 210: 299–305
143. Dürbeck HW, Klumpp E, Schladot JD, Schwuger MJ (1994) Environmental Specimen Bank of the Federal Republic of Germany—significance of surfactants. Progr Colloid Polym Sci 95:48–60
144. Karaolis-Danckert N, Buyken AE, Sonntag A, Kroke A (2009) Birth and early life influences on the timing of puberty onset: results from the DONALD (DOrtmund Nutritional and Anthropometric Longitudinally Designed) study. Am J Clin Nutr 90:1559–1565
145. Bourret P, Keating P, Cambrosio A (2011) Regulating diagnosis in post-genomic medicine: re-aligning clinical judgment? Soc Sci Med 73:816–824
146. Dunn RL, Huwe JK, Carey GB (2010) Biomonitoring polybrominbated diphenyl ethers in human milk as a function of environment, dietary intake, and demographics in New Hampshire. Chemosphere 80:1175–1182
147. Mir L (2009) Exposure to pesticides during pregnancy and neurobehavioral development of newborn babies. Environnement, Risque et Sante 8:92–93
148. Jurewicz J, Hanke W (2008) Prenatal and childhood exposure to pesticides and neurobehavioral development: review of epidemiological studies. Int J Occup Med Environ Health 21:121–132
149. Collins FS, Gray GM, Bucher JR (2008) Toxicology. Transforming environmental health protection. Science 319:906–907

150. Clewell HJ, Gentry PR, Covington TR, Sarangapani R, Teeguarden JG (2004) Evaluation of the potential impact of age- and gender-specific pharmacokinetic differences on tissue dosimetry. Toxicol Sci 79:381–393
151. Rostami-Hodjegan A, Tucker GT (2007) Simulation and prediction of *in vivo* drug metabolism in human populations from *in vitro* data. Nat Rev Drug Discov 6:140–148
152. Caudill SP (2010) Characterizing populations of individuals using pooled samples. J Expo Sci Environ Epidemiol 20:29–37

On the Role of Low-Dose Effects and Epigenetics in Toxicology

Lena Smirnova, Alexandra Sittka, and Andreas Luch

Abstract For a long time, scientists considered genotoxic effects as the major issue concerning the influence of environmental chemicals on human health. Over the last decades, a new layer superimposed the genome, i.e., the epigenome, tremendously changing this point of view. The term "epigenetics" comprises stable alterations in gene expression potential arising from variations in DNA methylation and a variety of histone modifications, without changing the underlying DNA sequence. Recently, also gene silencing by small noncoding RNAs (ncRNAs), in particular by microRNAs, was included in the list of epigenetic mechanisms. Multiple studies *in vivo* as well as *in vitro* have shown that a multitude of different environmental factors are capable of changing the epigenetic pattern as well as miRNA expression in certain cell types, leading to aberrant gene expression profiles in cells and tissues. These changes may have extensive effects concerning the proper gene expression necessary in a specified cell type and can even lead into a state of disease. Especially the roles of epigenetic modifications and miRNA alterations in tumorigenesis have been a major focus in research over the last years. This chapter will give an overview on epigenetic features and on the spectrum of epigenetic changes observed after exposure against environmental chemicals and pollutants.

Keywords Epigenome · Small noncoding RNAs · MicroRNAs · DNA methylation · Histone modification · Metallotoxins · Endocrine disruptors · Bisphenol A · Diethylstilbestrol · Vinclozolin · Particulate matter · Pharmaceuticals · Peroxisome proliferators · Ethanol · Dioxin · Benzopyrene

L. Smirnova, A. Sittka and A. Luch (✉)
Department of Product Safety, German Federal Institute for Risk Assessment, Max-Dohrn-Strasse 8-10, 10589 Berlin, Germany
e-mail: Andreas.Luch@bfr.bund.de

A. Luch (ed.), *Molecular, Clinical and Environmental Toxicology*,
Experientia Supplementum 101, DOI 10.1007/978-3-7643-8340-4_18,

Introduction

Throughout life span, humans are exposed to a variety of chemicals and other substances via diet, breathable air, and skin contact. In addition to inevitable sources of chemical exposure, individuals might subject themselves to alcohol, tobacco smoke, and other substances like cosmetic products, jewelry, and so forth. Also the uptake of pharmacological agents contributes as a major factor. The list of sources for chemical exposures is wide. Even though very often only present in slight traces, the exposure on a sustained basis may have enormous effects on human health. It is more and more observed that this influence is carried out not only through mutagenesis or physical damage to DNA but also on the level of epigenetic changes. If there have been any qualms to what extent the epigenome of humans underlies the influence of xenobiotics, the cohort study of Fraga and colleagues published in 2005 on epigenetic differences arising during the lifetime of monozygotic twins at the latest has resolved all doubt [1]. Monozygotic twins provide an elegant model to compare genetically identical individuals. In the study of Fraga *et al.*, 80 volunteer twins ranging from 3 to 74 years were investigated for their epigenetic patterns mostly of lymphocyte cells, concerning global DNA methylation and histone 3 (H3) and 4 (H4) acetylation. While 3-year-old twins showed very moderate, if any at all, differences in their global epigenetic profiles, the comparison of 50-year-old twins revealed major variations (concerning global DNA methylation as well as H3 and H4 acetylation). Differences in the epigenetic profile lead to variance in gene expression patterns. Global microarray analysis of protein-coding genes of the aforementioned twin pairs correlated extremely well with the analysis of the epigenetic profiles, showing a much higher variance in gene expression in the older twins as compared to young children.

A new class of noncoding regulatory molecules has been recently discovered that includes small interfering RNAs (siRNAs), microRNAs (miRNAs), and PIWI-interacting RNAs (piRNAs). Based on the immense increase of scientific studies published in the last years in this field, the role of small noncoding RNAs (ncRNAs) in the regulation of core cellular processes is no more in doubt. ncRNAs regulate a diverse spectrum of genes at the posttranscriptional as well as at the transcriptional level. Their mode of action involves, among others, key epigenetic modifiers: ncRNAs may either regulate translation of epigenetic enzymes (by miRNAs) or act in a complex with epigenetically active proteins in gene promoter regions at the DNA and chromatin level. Additionally, miRNA gene expression itself is often subject of epigenetic regulation (reviewed in [2]). The interplay of canonical epigenetic mechanisms and small ncRNAs contributes to regulation of cellular physiology and, as a result, may play a significant role in the cellular response to toxicants. Thus, the exposure to epigenetically effective substances can lead to alterations of a bright spectrum of regulatory mechanisms at the transcriptional and posttranscriptional level.

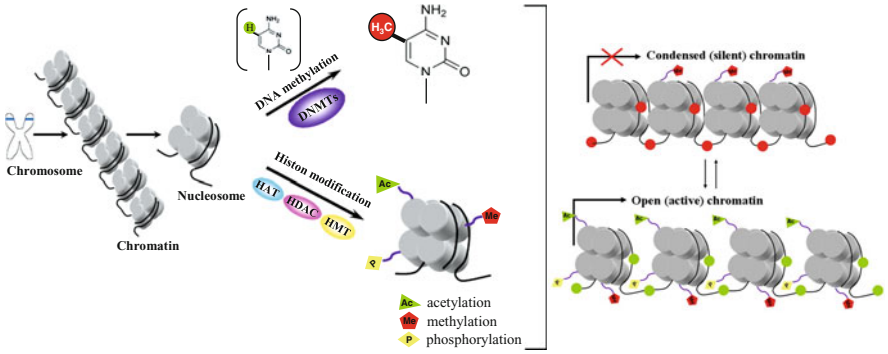


Fig. 1 Chromatin-related mechanisms of epigenetics: DNA methylation and histone modifications (see text for further details). *HAT* histone acetyltransferase, *HDAC* histone deacetylase, *HMT* histone methyltransferase, *DNMT* DNA methyltransferase

Epigenetic Features and the Complex Network of Participating Enzymes and Cofactors

Epigenetic regulation is based on two forms of modifications: modification of cytosine bases as well as posttranslational histone modifications (Fig. 1). Especially, regulatory and differentiation processes are highly susceptible to epigenetic alterations (i.e., genomic imprinting, X-chromosome inactivation, transposon silencing) [3, 4]. Epigenetic programming during development enables the establishment of different gene expression patterns, necessary for complex, multicellular organisms.

DNA Methylation

The modification on the DNA level is carried out by methylation at the C5 position of cytosines in the context of CpG dinucleotides, leading to the "5th base" 5-methylcytosine. This methylation process is conferred by DNA methyltransferases (DNMTs) (reviewed in [5]). Most of CpG dinucleotides randomly spread in the human genome exist in a methylated state (~90%), while those clustered in so-called CpG islands are mostly unmethylated, specifically when located in the promoter region of housekeeping genes [6, 7]. The class of mammalian DNMTs comprises enzymes with different specificities: maintenance methylation by DNMT1 and *de novo* methylation by DNMTs 3A and 3B (reviewed in [8]). The DNMT1 methylates the newly synthesized DNA strand during DNA replication according to the methylation pattern of the parental strand. This process provides the possibility of heredity transmission of methylation patterns from one cell to the daughter cells during cell division. The DNMTs 3A and 3B methylate cytosine bases in the context of CpG dinucleotides especially in CpG regions that have not

been methylated throughout the process of replication. The existence of *de novo* methylation enzymes renders DNA methylation into a highly dynamic process. In addition, in mammals, there is evidence that DNA demethylation occurs via active genome-wide demethylation in the male pronucleus immediately after fertilization and passive DNA demethylation after embryo development. A second round of demethylation occurs in primordial germ cells (reviewed in [9]). The search for enzymes containing demethylating activity revealed a 5-methylcytosine DNA glycosylase along with T–G mismatch glycosylase activity exerted by a thymine DNA glycosylase (TDG) in chicken [10, 11]. Also the methyl-CpG-binding protein MBD4 has been suggested to exert 5-methylcytosine in addition to thymine DNA glycosylase activity. However, 5-methylcytosine DNA glycosylase activity *in vivo* by both enzymes has not been proven yet.

While methylated cytosines are often correlated with a highly condensed chromatin structure and lead to gene silencing (Fig. 1), unmethylated cytosines are mainly found in relaxed chromatin sections and promote gene expression [12, 13]. However, this correlation accounts for only 30% of human promoters. The existence of silenced promoters showing no DNA methylation and the expression of methylated genes [14] suggests a combinative regulatory action of multiple components, aside DNA methylation, to promote gene silencing/activation.

Histone Modification

In addition to DNA methylation, histone modifications play a central role in epigenetic regulation. 146 base pairs of DNA are wrapped around a multicomponent protein core, consisting of two subunits of each of the different histones H2A, H2B, H3, and H4. This octamer is associated with one subunit of H1 [15]. These structures, the so-called nucleosomes, represent the building blocks of the higher order chromatin (Fig. 1). The N-terminal tails of the core histone proteins are subjected to a variety of different posttranslational modifications (methylation, acetylation, phosphorylation, ubiquitination, sumoylation, biotinylation), with methylation [16] and acetylation [17] being the best studied so far. While histone acetylation (mainly at multiple residues in the N-terminal tails of histones H3 and H4) is often found in open chromatin conformations (active chromatin, euchromatin), histone methylation—depending on the modified residues—is able to confer both a closed chromatin state (inactive chromatin, heterochromatin) and an open configuration. In general, there is a strong correlation between histone modification levels and the level of gene expression, making them a reliable informative value [18]. A fine and highly logistic network of a large variety of histone-modifying enzymes [histone acetyltransferases (HATs) and deacetylases (HDACs) (reviewed in [19]), histone methyltransferases (HMTs) [20], and demethylases [21–24], as well as others] creates a chromatin state for the entire genome specific for a given cell type in a specific proliferation state.

Cofactors

Histone- and DNA-modifying enzymes often interact with other protein factors which recognize specific elements and guide the enzyme to its place of action. The maintenance DNA methyltransferase, i.e., DNMT1, is localized to replication foci through interaction with the proliferating cell nuclear antigen (PCNA) [25, 26]. Upon DNA methylation, methylcytosine-binding proteins, such as MeCP2 and MBD2, can bind to the modified regions and are then able to guide chromatin-modifying enzymes, for example, HDACs, to the methylated regions [12, 27]. Chromatin states are additionally often reinforced by binding of accessory proteins or protein complexes. Different proteins targeting repressive chromatin-modifying enzymes to specific loci are compiled in the group of polycomb group proteins (PcGs) (reviewed in [28]). These proteins are antagonized by the trithorax group of proteins (TrxGs), which are required for active gene transcription. Both protein types bind to PcG/TrxG responsive elements (PREs/TREs) (reviewed in [29]). Different works have shown by now that some PcGs as well as TrxGs themselves contain histone modification activity. The PcG protein enhancer of zeste homolog 2 (EZH2), for example, was shown to contain a so-called SET (suppressor of variegation, enhancer of zeste, and trithorax) domain (characteristic for HMTs) and targets residue K27 in the histone tail of H3 [30, 31], thereby setting one of the most repressive marks with H3K27 methylation. The mammalian TrxG protein ALL-1/MLL was shown to be active in H3K4 methylation [32, 33], a characteristic mark of open chromatin, harboring transcriptionally active genes.

Based on the diversity of enzymes and cofactors involved in epigenetic regulation, it does not come as surprise that environmental chemicals and pollutants may exert the ability to disturb this fine-tuned regulatory network at multiple stages.

Noncoding RNAs

Small noncoding RNAs (ncRNAs) play a significant role in the epigenetic regulation of gene expression. This includes essential mechanisms like X-chromosome inactivation, heterochromatin formation, imprinting, and gene silencing. The most abundant classes of small ncRNAs are siRNAs, miRNAs, and piRNAs.

siRNAs are 20–23 nucleotide (nt) molecules, which are derived from long double-stranded RNA (dsRNA) transcripts through cleavage by the RNase III Dicer. siRNAs are subdivided into different subclasses, according to their origin: (1) siRNAs transcribed from repeat-associated genomic regions (rasiRNAs) are subsequently rearranged to piRNAs; (2) siRNAs from transposable elements (TE-siRNAs); (3) siRNAs from *cis* and *trans* natural antisense transcripts (*cis*-NAT-siRNA and *trans*-NAT-siRNAs); (4) siRNAs from hairpin RNAs which originate from extended inverted repeats; and (5) secondary siRNAs (2º siRNAs) which are synthesized by RNA-dependent RNA polymerase in the presence of primary siRNAs and their target transcript (identified in plants and worms) [34, 35]. siRNAs

were originally identified as exogenously introduced dsRNAs which mediate the RNA interference (RNAi) pathway. Then endogenous siRNAs have been described in plants, fungi, nematodes, and very recently in flies and mammals [34]. The main function of siRNAs is posttranscriptional gene silencing (PTGS) through cleavage of their target mRNA. siRNAs are involved in the regulation of heterochromatin structures and the defense of genome integrity in response to transposons, viruses, and transgenes (reviewed in [35–37]).

miRNAs constitute another large class of evolutionary conserved small ncRNA molecules (20–23 nt in length) that are derived from 70 nt-stem-loop precursors through cleavage by Dicer and are thought to act primarily as antisense inhibitors of target mRNA translation (Fig. 2; reviewed in [35, 38]). At present, the miRNA database miRBase 14.0 [39] comprises more than 10,000 entries, including miRNAs from diverse species throughout the plant and animal kingdoms; thus, miRNAs represent one of the largest gene families. Recent studies suggest that miRNAs may regulate the expression of more than 30% of the protein-coding genes in humans and other organisms [40, 41]. miRNAs participate in core regulatory processes with high time, cell, and tissue specificity, including proliferation, differentiation, apoptosis, organ development, and response to environmental stresses [42, 43]. Moreover, miRNAs have been shown to play a crucial role in diverse disease processes, including tumorigenesis [44], neurodegenerative disorders [45], diabetes [46], cardiac hypertrophy [47], and autoimmune diseases [48].

piRNAs are longer (28–33 nt) than canonical siRNAs and are expressed in the mammalian germ line. Scientists assume that piRNAs are processed from single-stranded (ss) transcripts, independently of RNase III Dicer, and may play a critical role in spermatogenesis through silencing of transposons. However, their exact mode of action is currently only poorly understood [50–52].

Posttranscriptional Gene Silencing by Small ncRNAs

siRNAs and miRNAs associate with the argonaute (Ago) protein family, a core component of the RNA-induced silencing complex (siRISC or miRISC), in order to silence target mRNAs by cleavage or translational repression, respectively (Fig. 2). miRNAs with nearly perfect complementarity to target sites direct mRNA cleavage, similar as siRNAs do [42]. miRISCs bind to 3′-untranslated regions (3′UTR) of target mRNAs at multiple binding sites with mismatches and bulges. While miRNAs usually act on *trans*-encoded transcripts, siRNAs direct the siRISC to a single perfect complementary binding site in an open reading frame (ORF), usually of the same gene locus the siRNA is derived from.

In contrast to siRNAs and miRNAs, piRNAs act through PIWI proteins, a subclass of the Argonaute protein family. piRNAs interact with PIWI proteins and induce posttranscriptional silencing of transposon elements through the so-called ping-pong cycle mode. Briefly, a primary antisense piRNA molecule which derives from a large piRNA cluster, containing transposon fragments, cleaves a

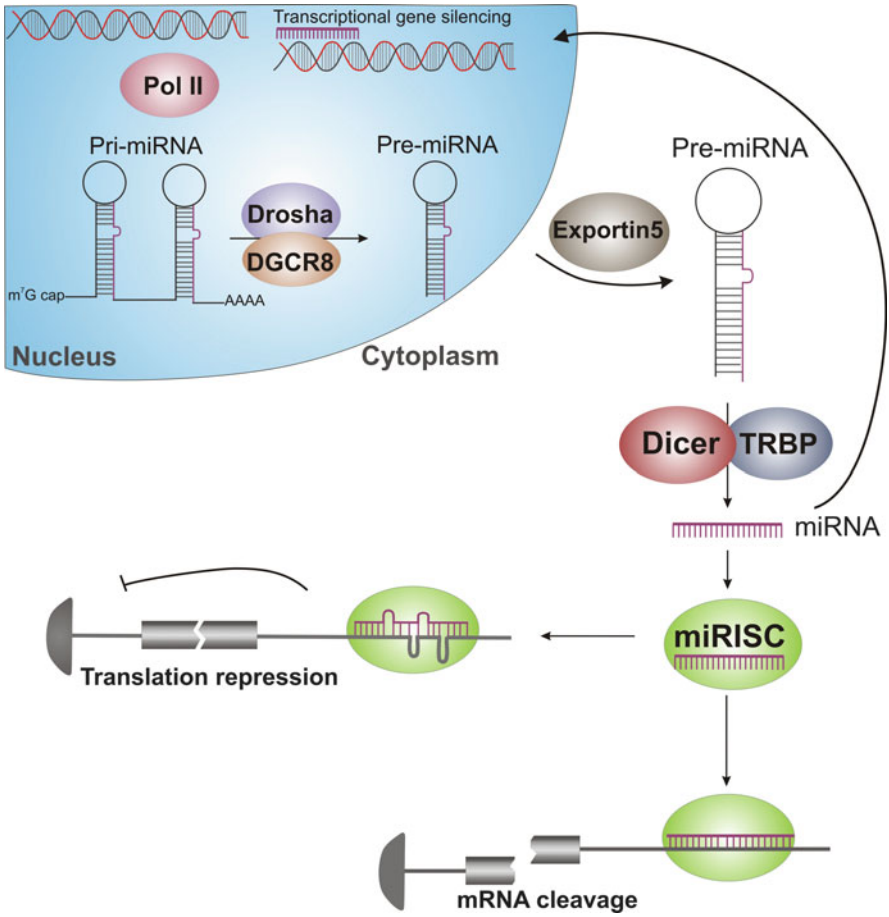


Fig. 2 MicroRNA biogenesis (adapted according to [49]). miRNAs are transcribed by RNA polymerase II into long primary transcripts (pri-miRNA). Pri-miRNAs are cleaved by RNase III Drosha into ~70 nt-stem-loop precursors (pre-miRNA) which are transported by Exportin 5 into the cytoplasm where they are then processed by RNase III Dicer into mature ~20 nt-long miRNAs. Mature miRNAs now assemble into the miRNA-induced silencing complex (miRISC) in order to repress translation in the case of imperfect complementarity between miRNA and target mRNA or to cleave mRNA when the miRNA perfectly fits to its target. Recently, it has been shown that miRNAs can be transported into the nucleus where they are involved in the regulation of gene transcription by targeting promoter regions (see text for further details). *TRBP* transactivating response (TAR) RNA-binding protein, m^7G 7-methylguanosine, *DGCR8* DiGeorge syndrome critical region gene 8, *Pol II* RNA polymerase II

transposon transcript. This initial cleavage generates a secondary sense piRNA which in turn cleaves an antisense transposon transcript from the piRNA cluster, creating a novel antisense piRNA that restarts the cycle through binding to a new transposon transcript [53, 54]. The detailed description of biogenesis and mechanisms of target recognition and repression by small ncRNAs is beyond the scope of this chapter and comprehensively described elsewhere [35–37, 42].

Transcriptional Gene Silencing by Small ncRNAs: The Role of miRNAs and siRNAs in Epigenetics

In addition to the well-known role of small ncRNAs in PTGS on the mRNA level, mostly all types of small RNAs may function at the DNA and chromatin level as well. A direct link between RNAi and heterochromatin formation has been best characterized in fission yeast (*Schizosaccharomyces pombe*) and plants (reviewed in [55–57]). It was shown that plant Ago4 protein is required for siRNA-directed DNA methylation and histone modification through interaction with RNA polymerase IV or V in order to silence mobile elements [58–60]. In *S. pombe*, the association of the RNAi machinery with histone-binding proteins results in the establishment of silenced heterochromatin where siRNAs are required to direct the histone-modifying enzymes to specific chromatin regions such as centromeric repeats [61, 62]. Depletion of Ago1, Dicer, or RNA-dependent RNA polymerase leads to reduction of H3K9 methylation and heterochromatin gene silencing [63].

Evidence for transcriptional gene silencing (TGS) by small ncRNAs in mammals has been steadily increasing. Several studies in mammalian cells have demonstrated an induction of heterochromatin formation upon transfection with siRNAs targeted to gene promoter regions, leading, in particular, to DNA and H3K9/H3K27 methylation. These findings were supported by the observation that RNAi core proteins, such as Ago1 and TRBP2, are associated with polymerase II and PcG EZH2 at the promoter regions of the siRNA-targeted genes [64–68]. The endogenously expressed miRNAs miR-320, miR-17-5p, and miR-20a were shown to direct TGS and chromatin remodeling by targeting the promoter regions of their target genes in mammalian cells [69, 70]. Regulation of transposon expression by piRNA was primarily thought to be posttranscriptional, but recent studies suggested also a transcriptional mechanism underlying transposon silencing. The hypothesis that piRNAs in complex with PIWI proteins participate in *de novo* DNA methylation of mobile elements in germ cells was supported by the finding that deficiency of two PIWI family members, *Mili* and *Miwi2*, leads to the loss of transposon DNA methylation and to a similar phenotype as observed in *Dnmt3l*-deficient mice [51, 52].

The precise mechanism of how small ncRNAs direct DNA methylation and histone modification remains mostly unclear. However, there is evidence supporting the model where small ncRNAs direct chromatin remodeling and TGS through the recognition of promoter-associated nascent RNA transcripts [66, 70, 71].

The Interplay of Epigenetics and miRNA

Considering the role of miRNAs in core physiological processes, it is obvious that their own expression needs to be tidily regulated. Several reports have suggested a temporal and spatial posttranscriptional regulation of miRNA stem-loop precursor processing [72–75]. In addition, a number of studies have shown that miRNA genes

undergo epigenetic alterations (reviewed in [2]). Regulation of miRNA gene expression by epigenetic mechanisms is best characterized and described during tumorigenesis [76]. So, during the last few years, increasing numbers of studies have been showing hypo- and hypermethylation of various miRNA genes in diverse cancer tissues and cell lines [77–79]. Moreover, epigenetic modifying drugs that are predominantly used in cancer therapy have been shown to alter miRNA expression as well [80, 81].

On the other hand, in addition to TGS by small ncRNAs described above, the key epigenetic modifiers have been predicted to be the targets for PTGS by miRNAs (see http://www.targetscan.org/vert_50/ and [41, 82]). Thus, Fabbri *et al.* have experimentally confirmed that *Dnmt3a* and *Dnmt3b* are target genes for the miR-29 family [83, 84]. Unexpected was a demonstration that miR-148 targets *Dnmt3b* not in its 3′UTR, contrary to the classic model of miRNA action, but in its protein-coding region [85]. HDAC4 was shown to be translationally regulated by cartilage-specific miR-140 or muscle-specific miR-1/miR-206 during bone development and myoblast differentiation, respectively [86–88]. miR-10a has been found to target HDAC4 during smooth muscle differentiation of mouse embryonic stem cells [89]. Retinoblastoma-like 2 (RBL2) protein, a posttranscriptional repressor of DNMT3A and DNMT3B, is a target for embryonic stem cell-specific miR-290. Depletion of Dicer in embryonic stem cells leads to downregulation of miRNA processing, thereby causing a lack of miR-290-mediated *Rbl2* repression and consequently resulting in the decrease of DNMT activity [90].

Finally, small ncRNAs can be implicated in the inheritance of epigenetic information through initiation of paramutations that can reveal insights in the mechanisms of transgeneration toxicology (reviewed in [91]). Thus, miRNAs and canonical epigenetic mechanisms interact in a reciprocal circuit to regulate key cellular processes.

The Role of Epigenetic Mechanisms and miRNA in Toxicology

The call for epigenetic mechanisms underlying toxicological effects of substances arises specifically upon exclusion of mutagenicity. Genotoxic substances that might increase the risk for diseases have been filed and provide a platform for risk assessment [92]. The contribution of miRNAs and epigenetic regulation in various human disorders, in particular cancer, sheds new light on toxicology and makes them an interesting and exciting tool to study pathological processes and for the development of new therapeutic strategies. Cancer is the disease most commonly associated with aberrant DNA methylation and altered miRNA expression. A huge number of studies were carried out, investigating DNA methylation and miRNA expression differences between tumors and adjacent healthy tissues, trying to determine tumor-specific profiles. Aside observed typical hypomethylation patterns in tumor cells, the list of mismethylated promoters and altered miRNA expression is continuously expanding [93–95]. Taking into account that small ncRNAs, in particular miRNAs, regulate transcriptional and posttranscriptional silencing of a massive panel of genes and are

regulated themselves by canonical epigenetic mechanisms, it becomes clear that miRNAs are likely to play an essential role in the cellular responses to environmental stress, including xenobiotics. The observation that epigenetic modulators like nickel and arsenic are able to transform normal cells into tumor cells provided a direct link between epigenetically active substances and the development of cancer [96, 97]. Also, failure in the imprinting of genes (differential expression of maternally and paternally derived DNA) is known as the basis for several diseases (e.g., Beckwith–Wiedemann, Prader–Willi, and Angelman syndromes, Wilms tumor) [98]. Based on the aforementioned reasons, it is about time to include epigenetic modulator and miRNA activity in the basic principles of risk assessment.

Environmental Influence on Epigenetic Gene Regulation

As initially mentioned, studying the epigenetic profile of monozygotic twins has manifested the credo of the influence of the environment on the epigenetic state of individuals [1]. It was early observed that aging alone leads to an epigenetic shift in mice. In the late 1980s, Wareham and coworkers showed an increase in gene expression of a silenced X-linked enzyme (ornithine carbamoyltransferase) with increasing age of the mice investigated [99], thus confirming a study from the late 1970s of Cattanach, who was able to show age-related reactivation of the X chromosome with the help of an introduced autosomal gene [100]. Nowadays, also studies in mice on imprinted genes located on autosomes have revealed their reactivation in aging animals. The imprinted genes *Igf2* (insulin-like growth factor 2) and *Cdkn1c* (cyclin-dependent kinase inhibitor 1c), both located at 11p15.5, show reactivation of the silent allele [101, 102]. In general, massive DNA demethylation upon aging in mice has been shown by introduction of an intracisternal A-particle (IAP) retrotransposon [103].

Aside simple aging of cells, also environmental stimuli like nutrition are able to exert epigenetic modulatory effects. DNA methylation by DNMTs depends on the availability of the methyl-group donor *S*-adenosyl-methionine (SAM). Since folate, a coenzyme of one-carbon metabolism, is involved in the generation cycle of SAM, it has been assumed that nutrition might have an impact on the proper establishment and maintenance of DNA methylation patterns. The feeding of a folate-deficient diet to mice results in a global reduction of DNA methylation in the liver [104]. In addition, folate starvation during pregnancy leads to hypomethylation in the developing embryo. This has been shown using the agouti mouse strain, a line carrying an IAP sequence in the agouti gene, preceding the first exon and resulting in the "viable yellow" allele (A^{vy}) which leads in a hypomethylated (and therefore fully expressed) state to a yellow fur color—in addition to other symptoms like obesity, hyperinsulinemia, diabetes, increased somatic growth, and increased susceptibility to hyperplasia and tumorigenesis [105]. Upon methylation of the IAP sequence, transcription of the A^{vy} allele is shut down, resulting in pseudoagouti mice showing a brown fur color. Depending on the extension of DNA methylation, the fur color

allows the discrimination of yellow over light to heavily mottled fur up to the pseudoagouti phenotype, providing a neat model to study DNA methylation. The feeding of methyl-deficient diets to pregnant mice results in a clear shift toward the yellow phenotype of the offspring indicating hypomethylation of the IAP sequence [106, 107]. Therefore, epigenetic changes in humans during aging most likely depend, aside on decreased efficiency [108] as well as reduced expression of DNMT1 [109], also on declining folate availability and uptake [110]. A methyl-deficient diet fed to mice has been shown not only to lead to DNA hypomethylation but also to confer changes in histone methylation levels and miRNA expression [111–113], suggesting an influence of nutrition on the epigenetic status in multiple ways. The role of epigenetically active substances in toxicology has raised much attention over the last years, and a number of toxicological agents have been investigated with respect to their epigenetic activity.

Alteration of miRNA Expression as a Cellular Response to Environmental Stress

It has been shown that miRNA expression is altered as a result of oxidative stress, cold stress, and under hypoxia or nutrient deficiency. Accordingly, Simone and coworkers have shown global changes in miRNA gene expression profiles in human fibroblasts upon oxidative genotoxic stress induced by anticancer agents such as ionizing radiation, hydrogen peroxide (H_2O_2), or etoposide [114]. The pretreatment of the cells with antioxidant cysteine prevented production of reactive oxygen species (ROS) and reduced irradiation-mediated alterations in miRNA expression, suggesting the role of miRNA genes in cellular defense against genotoxic oxidative stress. Antiapoptotic miR-21 was also shown to be sensitive to H_2O_2 stimulation in cardiac myocytes. H_2O_2-induced cell death was increased after treatment of the cardiomyocytes with miR-21 inhibitors or by ectopic overexpression of a miR-21 target, the programmed cell death 4 (*Pdcd4*) gene, while overexpression of the miR-21 precursor, i.e., pre-miR-21, significantly decreased apoptosis, suggesting an essential role of miR-21 in coordination of the protective response to oxidative stress induced by ROS [115]. The influence of ionizing irradiation on miRNA expression was supported by a variety of studies. Some miRNAs (miR-125a, miR-189) have been shown to exert radioprotective effects, while others (miR-127, let-7a/g, miR-181a) contribute to an enhanced radiosensitivity of the different cell types [116–119].

Under hypoxic stress, an alteration in the translation of a variety of genes is mediated among others by miRNAs (reviewed in [120, 121]). Wilmink and colleagues have detected thermally regulated miRNAs, expression of which was changed under hyperthermal environmental conditions [122]. Hypothermia was also shown to influence the miRNA profile through RBM3, an RNA-binding protein which regulates translation in response to hypothermia as an RNA chaperone [123].

Nutrient deprivation or redundancy is able to alter miRNA expression as well. Folate deficiency for 6 days was shown to induce a global increase of miRNA expression in human lymphoblastoid cells [124]. In particular, miR-222 was shown to be significantly overexpressed under folate starvation, a fact that was confirmed *in vivo* in human blood cells from individuals with folate-deficient diet. The authors could not observe any changes in global DNA methylation levels, and the alterations in the expression of all miRNAs observed *in vitro* were reversible as cells were returned to folate-completed medium and incubated for further 10 days. These data suggest that miRNA alterations occur before irreversible genetic or epigenetic effects are being established, and chronic, continuous exposure is possibly required to maintain miRNA changes [124]. Moreover, miRNAs can induce chemokine production as a response on serum starvation in cultured cells [125, 126]. Geadicke's group has demonstrated the changes in hepatic miRNA expression *in vivo* upon deficiency or sufficiency of vitamin E in rat diet [127]. Interesting was the observation that maternal high-fat diet during pregnancy and lactation has altered the expression of liver-specific miRNAs in offspring [128].

One of the consequences of chronic environmental stress or long-term effects of toxicants could be an invariable alteration of gene expression (including miRNA genes) and epigenetic marks and, as a result of both, changes in cellular physiology that can initiate the development of diseases.

Chemicals as Modulators of Epigenetic Regulation and miRNA Expression

Aside nutrition, individuals encounter reams of environmental stimuli over their life span, and the performance of epidemiological studies should help to fill the gap between environmental factors and the development of diseases and possible underlying epigenetic mechanisms. Even though the literature concerning epigenetic alterations and their relation to certain diseases is always expanding, investigations concerning the development of cancer and the putative involvement of environmental factors have for sure the longest history in research.

Since endogenously expressed siRNAs as well as piRNAs have been discovered very recently, there are only limited data so far about their participation in toxicological processes. In contrast, miRNAs were discovered a decade ago as the most widespread class of small ncRNAs. Therefore, the knowledge about their role in toxicology and cellular responses to environmental stress is becoming more and more clear. Tissue- and organ-specific expression patterns of miRNAs, as well as altered miRNA profiles in tumorigenesis, suggest that miRNA expression may change in response to xenobiotics (Table 1). This can provide toxicant-specific profiles, opening a new role of miRNAs as potential toxicological biomarkers. Several research groups in the field of environmental health have used miRNA profiling to elucidate effects of toxic substances on miRNA expression. In view of the fact that miRNA genes and epigenetic enzymes are

Table 1 Chemical-induced alterations of miRNA expression

Agent	Species	miRNA	Targets/function	References
Arsenic	Human	↑ miR-222, ↓ miR-210	SAM metabolism, changes in genomic methylation	[124]
	Human	↓ miR-200a/b/c	ZEB1,2/epithelial–mesenchymal transition	[169]
	Chicken	↓ miR-9, miR-181b	NRP1/angiogenesis	[171]
	Human	↓ miR-19	PTEN/induction of apoptosis	[172]
Aluminum	Human	↑ miR-146	CFH/inflammation	[203]
	Human	↑ miR-9, miR-125, miR-128	Neurotoxicity	[204]
BPA	Human	↑ miR-146	Reduction of proliferation	[215]
DES	Human	↓ miR-9-3c	TP53/apoptosis, breast cancer development	[225]
Metal-rich PM	Human	↑ miR-222, miR-21	Response to oxidative stress and inflammation	[253]
	Rat	↓ miR-1, miR-133, miR-21, miR-24, miR-29	Heart failure, cardiac hypertrophy, and fibrosis	[253, 257]
Cigarette smoke	Human	↓ miR-218	MAFG/modulation of gene expression	[273]
	Rat	↑ miR-294	ZNF697, ARID4A/global increase of gene transcription	[268]
	Rat	↓ let-7, miR-10, miR-145, miR-146, miR-222	Stress response, apoptosis, proliferation, angiogenesis	[270]
NNK	Rat	↓ miR-34, miR-101, miR-126, miR-199	TP53, CYP2A3/apoptosis, tumorigenesis	[275]
VPA	Rat	↓ miR-34a	GRM7/neurotransmission	[282]
Lithium	Rat	↓ let-7b,c, miR-128, miR-24a, miR-34a	Potential targeted pathways: PTEN, axonal guidance, ERK, WNT/β-catenin	[282]
	Human	↑ miR-34a, miR-152, miR-155, miR-221		[282, 283]
Paracetamol, CCl_4	Rat	↓ miR-298, miR-370	Thioredoxin reductase 3/oxidative stress, hepatotoxicity	[284]
Tamoxifen	Rat	↑ miR-34, miR-17-92, miR-106a	RB1/hepatocarcinogenesis	[291]
2-AAF	Rat	↑ miR-31	CEBPA/hepatocarcinogenesis	[293]
Peroxisome, proliferators	Mouse	↓ let-7c, ↑ miR-17-92	c-MYC/proliferation, hepatocarcinogenesis	[296]
5-Aza-2′-deoxycytidine	Human	↑ miR-127, miR-34b/c	BCL6, BTG4/tumor suppression	[80, 81]
5-FU	Human	↑ miR-200b	PTPN12/inactivation of oncogenes	[298]
Hexogen (RDX)	Mouse	↑ miR-206, miR-195, miR-30a	BDNF/neurotoxicity	[299]

(*continued*)

Table 1 (continued)

Agent	Species	miRNA	Targets/function	References
	Mouse	↑ miR-17-92, miR-15-16, miR-181, miR-26, let-7, ↓ miR-10b	Tumorigenesis, regulation of cell cycle	[299]
Ethanol	Mouse	↑ miR-9	BK ion channel/ethanol tolerance, neurotransmitter release, synaptic plasticity	[304]
	Human	↑ miR-212	ZO1/permeability of intestinal barrier	[305]
	Mouse	↓ miR-21, miR-335, miR-9, miR-153	Jagged-1, ELAVL2/induction of cell cycle and neuroepithelial cell proliferation	[306]
	Mouse	↑ miR-10a/b	HOXA1/pathogenesis of fetal alcohol syndrome	[308]
TCDD	Human	↑ miR-191	SOX4/hepatocarcinogenesis	[311]
BPDE	Human	↓ miR-10a	HOXA1/malignant transformation of cells	[313]

↑ upregulation, ↓ downregulation, *2-AAF* 2-acetylaminofluorene; *ARID4A* AT-rich interactive domain 4; *BDNF* brain-derived neurotrophic factor; *BK* big potassium (ion channel); *BPA* bisphenol A; *BPDE anti*-benzo[*a*]pyrene-7,8-diol-9,10-epoxide; CCl_4 carbon tetrachloride; *CEBPA* CCAAT enhancer binding protein α; *CFH* complement factor H; *DES* diethylstilbestrol; *CYP2A3* cytochrome P450-dependent monooxygenase 2A3; *ELAVL2* embryonic lethal, abnormal vision, *Drosophila*-like 2 (protein); *5-FU* 5-fluorouracil; *GRM7* metabotropic glutamate receptor 7; *MAFG* v-maf musculoaponeurotic fibrosarcoma oncogene homolog G; *NRP1* neuropilin 1; *PM* particulate matter; *PTEN* phosphatase and tensin homolog (protein); *PTPN12* protein tyrosine phosphatase 12; *NNK* 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; *RB1* retinoblastoma (protein) 1; *RDX* Research Department Explosive/Royal Demolition Explosive (i.e., hexahydro-1,3,5-trinitro-1,3,5-triazine, hexogen); *SAM S*-adenosyl-methionine; *SOX4* SRY (sex determining region)-related HMG-box (protein); *TCDD* 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; *TP53* tumor (suppressor) protein 53; *VPA* valproic acid; *ZEB1,2* zinc-finger E-box binding homeobox factors 1 and 2; ZNF697, zinc-finger protein 697; *ZO1* zonula occludens 1 (protein)

interrelated, the most interesting question to be answered is if the known epigenetic active substances may influence miRNA biogenesis.

Epigenetic Activity of Metallotoxins

Nickel

As early as during the first decades of the nineteenth century, nickel has been identified and investigated as a toxicological compound. In 1882, Stuart published a comprehensive animal study on the toxicological action of nickel and cobalt in different animal models [129]. In the last century, nickel gained increasing attention based on the observation of nickel belonging to the class of contact allergens. Today, a carcinogenic effect of nickel has been well established. Ever since the first hints that nickel exerts its effects through epigenetic modulator activity [130, 131], multiple labs tried to decipher the exact mode of action. It was early observed that nickel ions predominantly interact with heterochromatic chromosomal regions. The treatment of Chinese hamster ovary (CHO) cells with nickel sulfide was shown to selectively damage the heterochromatic long arm of the X chromosome, whereas the euchromatic short arm was left unaffected [132–134]. Further studies were carried out to unravel the epigenetic effects exerted by nickel to explain its carcinogenicity without exhibiting a strong genotoxic impact. It was shown that increasing concentrations of magnesium ions (Mg^{2+}) lead to a protection against nickel-induced transformation and DNA damage [133], and an *in vitro* study of Ellen and coworkers revealed the replacement of Mg^{2+} ions in heterochromatin by Ni^{2+} ions, leading to an enhanced chromatin condensation [135]. This provides an explanation for the earlier observation of the silencing of a *gpt* reporter gene by DNA methylation in the presence of nickel when introduced into a heterochromatic DNA region in the Chinese hamster cell line G12 [136]. Following magnesium replacement by nickel ions and an increased chromatin condensation, subsequent DNA methylation of the heterochromatic region is very likely to occur, leading to silencing of the underlying gene sequences. Govindarajan and colleagues were able to show methylation and silencing of the tumor suppressor gene $p16^{Ink4a}$ in tumor tissue developed in mice after implantation of nickel sulfide [137], providing a new piece for the puzzle of nickel carcinogenesis. Additionally, the analysis of DNA repair genes in nickel-treated as well as nickel-transformed cell lines revealed methylation of the CpG island and transcriptional downregulation of the O^6-methylguanine DNA methyltransferase (*Mgmt*) gene promoter [138], a feature often observed in tumor tissues [139, 140]. Recently, the correlation between nickel subsulfide (Ni_3S_2) exposure and hypermethylation of 5′ regions of *Rar-β2*, *Rassf1a*, and *Cdkn2a* tumor suppressor genes and accompanying decreasing mRNA levels was described in tumors in Wistar rats [141].

The exposure to nickel compounds influences not only DNA methylation but also histone modifications. The exposure of human lung epithelial A549 cells to nickel chloride results in reduced acetylation of histones H2A, H2B, H3, and H4.

In addition, a significant increase in H3K9 dimethylation (H3K9me2), H3K4 trimethylation (H3K4me3), as well as in H2A and H2B ubiquitination can be observed [9, 142–145]. While increased ubiquitination upon nickel exposure could be attributed to inhibition of deubiquitination [146], the increase in H3K9 methylation is probably exclusively due to inhibition of the histone demethylase JMJD1A [147], rather than the result of an increase in histone methylation. This conclusion is supported by the observation that methyltransferase G9a is strongly downregulated at the protein level in nickel-treated cells [144, 147]. The data obtained over the last years provide strong evidence for an epigenetic mechanism underlying the carcinogenic effects of nickel that has been claimed decades ago based on the observation of lung and nasal cancer in nickel workers [148–150].

Arsenic

Arsenic constitutes another example of a highly toxic environmental contaminant that has been proven to exhibit epigenetic modulator activity (reviewed in [151]). There is evidence from epidemiological studies that exposure to arsenic increases the risk of cancer. An occupational exposure to arsenic by inhalation leads to an increased risk of lung cancer [152–154]. Aside occupational exposures, arsenic can be found in water, soil, and airborne particles, as well as in foods. The exposure to arsenic usually occurs in the form of arsenite [As(III)] or arsenate [As(V)], with arsenite showing the higher toxicological potential [155]. By now, different groups have demonstrated that long-term exposure of mammalian cells to low doses of arsenic leads to malignant transformation *in vitro* [94, 156, 157]. One major aspect of the toxicological and carcinogenic action of arsenic is based on its ability to interfere with the DNA methylation process. Exposure to low doses of arsenite leads to global hypomethylation in mammalian cells in a time- and dose-dependent manner [96]. This demethylating effect of arsenite is exhibited through interference with SAM, which is used in mammalian cells as the methyl-group donor in DNA and histone methylation reactions (cf. above). Arsenite becomes detoxified through methylation by SAM, serving also here as the methyl donor (involved methyltransferases are currently unknown). Exposure even to low doses of arsenite subsequently leads to the consumption of SAM, resulting in increased levels of *S*-adenosyl-homocysteine (SAH) and therefore to a reduced SAM/SAH ratio in treated cells [158]. In addition, downregulation of the major DNA methyltransferases *DNMT1* and *3A* at the mRNA level has also been shown. Aside global hypomethylation upon arsenite treatment, also gene-specific promoter hypermethylation can be observed. Human lung adenocarcinoma cells exposed to arsenite exhibit hypermethylation of the promoter of *p53* (tumor protein 53, TP53) [159]. This hypermethylation of the tumor suppressor gene *p53* promoter was also observed in people exposed to arsenic when compared to control persons [160]. In addition, arsenic-induced hypermethylation of promoters of tumor suppressor genes, such as $p16^{Ink4a}$, *Rassf1a*, *Prss3*, and *Dapk* (death-associated protein kinase), could be observed in different cancer samples [161–163].

Arsenic influences not only DNA methylation but also histone modifications. Ramirez *et al*. showed that exposure of HepG2 cells to arsenite leads to a global increase in histone acetylation, mostly due to inhibition of HDACs [164]. Promoter-specific histone acetylation in response to arsenic treatment had been already described earlier by Li and colleagues [165, 166]. Conversely, Jo *et al*. have observed a dose- and time-dependent reduction in the global levels of H4K16 acetylation in human bladder epithelial cells exposed to arsenite [167]. Recent work from Costa's lab also described an increase in H3K9me2 and H3K4me3 levels in lung adenocarcinoma cells upon arsenic treatment [9, 168]. This is in contrast to the work of Ramirez *et al*. who were unable to detect this effect in HepG2 cells [164]. Taking together, further investigations are necessary for a better understanding of the mechanisms of arsenic-induced histone modifications. Nevertheless, there is no doubt that arsenic exerts its epigenetic modulator activity on both the DNA level and the histone level.

In addition, exposure of human lymphoblastoid cells to sodium arsenite led to global increases of miRNA expression [124]. Interesting was the observation that arsenic exposure led to alterations of the same miRNAs that were altered by folate deficiency, supporting the idea that arsenic may act by altering the one-carbon metabolism. No changes in global methylation patterns were observed under these exposure conditions. Thus, the arsenic-induced changes in cellular miRNA levels may occur before any global epigenetic changes may supplement already known epigenetic effects of arsenic.

Wang and coworkers have shown that long-term exposure of TP53 knockdown human bronchial epithelial cells to low doses of arsenite causes malignant transformation of the cells along with downregulation of miR-200 family expression, key regulators of the epithelial–mesenchymal transition [169, 170]. The authors could demonstrate that mir-200b plays a protective role against malignant transformation induced by arsenite. Most recently, it has been shown that sodium arsenite may induce angiogenesis by altering the expression of miR-9 and miR-181b, both of which target neuropilin 1 (NRP1), a transmembrane receptor involved in vascular development [171].

Contradictory to the well-established role of arsenic as a carcinogenic agent, oncogenic miR-19a was found significantly downregulated in T24 human bladder carcinoma cells after exposure to arsenic trioxide [172]. Direct target of miR19a, PTEN, a well-known tumor suppressor, was upregulated upon both arsenic trioxide exposure or miR-19a depletion. These results provide a new possible mode of action of arsenic in human cancer cells.

Chromium

Chromium is usually taken up in the hexavalent form [Cr(VI)] which emerges as side product of industrial processes, upon burning of fossil fuels and waste or which can be elutriated from chromium-containing waste. Upon ingestion or respiratory uptake, Cr(VI) is reduced, leading to the end product of metabolism in mammalian cells,

trivalent chromium [Cr(III)], the latter representing a thermodynamically stable state [173, 174]. Chromium is an agent well known to cause respiratory cancers upon occupational exposure (reviewed in [175]). Aside acting as a DNA damaging and mutagenic substance, several reports over the last decade have demonstrated epigenetic effects exerted by chromium. Klein and coworkers reported in 2002 on the ability of soluble chromate salts to induce hypermethylation of the *gpt* transgene in Chinese hamster G12 cells [176], a system that had been used earlier to show epigenetic effects of nickel, arsenic, and other compounds. Treatment of the G12 cell line as short as 2 h in the presence of potassium chromate (K_2CrO_4) led to partial methylation of the *gpt* gene, as analyzed by southern blots. By contrast, treatment using the insoluble barium chromate ($BaCrO_4$) did not induce any methylation changes. In an epidemiological study conducted in 2006 by Kondo *et al.*, the investigation of lung tumor tissues from chromate workers compared to lung tumors of patients that had not been occupationally exposed revealed a higher incidence of methylation of the $p16^{Ink4a}$ promoter region in chromate lung cancers compared to the control tumors, even though the difference was not significant [177]. However, the analysis of lung tumor tissues of chromate workers with respect to the time period of occupational exposure showed a significant increase of $p16^{Ink4a}$ promoter methylation with increasing exposure time. While promoter methylation could not be detected in tumor tissues of workers that were classified as shortly exposed (<15 years), the incidence of promoter hypermethylation of the $p16^{Ink4a}$ gene increased to 43% and 40% in workers exposed to chromium for a moderate (15–24 years) and long time (>25 years), respectively [177]. When correlating promoter methylation to protein expression, there was a clear tendency of chromate lung cancer with methylation of the $p16^{Ink4a}$ gene to show reduced expression of the $p16^{INK4A}$ protein. With regard to the molecular mechanism underlying chromate-induced epigenetic variation, not much is known to date. Work from the Puga laboratory during the last decade revealed a function for chromium in the protein cross-linking to chromatin, resulting in inhibition of histone remodeling marks. These results are based on the observation of a lack of cytochrome P450-dependent monooxygenase (CYP) 1A1 expression upon binding of ligands (dioxin and benzo[*a*]pyrene) to the aryl hydrocarbon receptor (AHR) in the presence of chromium [178, 179]. The loss of induction could be attributed to a block of HDAC release and therefore inhibition of HAT recruitment [179]. A more recent publication in 2007 showed that chromium exposure leads to the formation of HDAC1–DNMT1–chromium–chromatin complexes at the proximal promoter of the *Cyp1a* gene, thereby inhibiting the recruitment of RNA polymerase II as well as p300, factors of the basal transcription complex [180]. Epigenetic silencing upon chromium treatment was also shown for the *hMlh1* gene, which is involved in DNA mismatch repair. Here, epigenetic alterations were shown on the DNA as well as on the histone modification level. Chromium leads not only to an increased DNA methylation in the promoter region of the *hMlh1* gene in tumors of chromate workers [181] but also to an increase in silencing H3K9me2 [182], resulting in a reduced *hMlh1* mRNA level in mammalian cells *in vitro*. In addition, Sun and colleagues were able to show that chromium also leads to alterations in the global H3K9, H3K27, H3K4, and H3R2 methylation levels [182].

About a decade ago, biotin, which is an essential coenzyme for carboxylases in mammals, was discovered to bind to histones, thus suggesting a role of this vitamin in histone modification [183]. Two enzymes are responsible for histone biotinylation: biotinidase and holocarboxylase synthetase [184, 185]. Very recently, it was shown that Cr(VI) decreased the levels of biotinidase mRNA in human bronchial epithelial 16HBE cells after 24-h treatment [186]. This effect was reversed by treatment of the cells with the HDAC inhibitor trichostatin A, suggesting that chromate may reduce the level of biotinidase by modification of histone acetylation [186]. Further investigations are needed to evaluate possible effects of chromium on histone biotinylation.

Cadmium and Methylmercury

Based on its wide use in industry, cadmium is a heavy metal affecting humans through occupational as well as environmental exposures. Cadmium is present in soils, sediments, air, and water. Several studies assigned cadmium a role in human pulmonary, prostate, and renal cancers (reviewed in [187, 188]). Even though a study of Filipič and Hei indicated a role of cadmium in mutagenicity [189], it was shown to be inactive in bacterial test systems and only weakly mutagenic in mammalian cell mutagenicity assays [190]. Concerning epigenetic changes induced by cadmium, it could be shown that cadmium exposure leads to a reduced DNMT activity and DNA methylation level in human cells [191, 192]. DNA hypomethylation resulted in a strong increase in cell proliferation [192]. Longtime exposure *in vitro* even resulted in cell transformation, unexpectedly accompanied by DNA hypermethylation and increased DNMT activity [191, 193]. In 2007, Benbrahim-Tallaa *et al.* were able to correlate DNA hypermethylation in cadmium-transformed cells to an increase in the expression level of the *de novo* DNA methyltransferase DNMT3B. The increase in DNMT3B expression leads to promoter methylation of the tumor suppressor genes $p16^{Ink4a}$ and *Rassf1a* in cadmium-induced malignant transformation of human cells [194]. Subchronic feeding of hens with cadmium revealed an increase in DNA methylation levels and expression of DNMT1 and 3A, but not 3B in liver and kidney [195]. Most recently, changes in the levels of DNA methylation in testis of rats neonatally exposed to cadmium were observed by Zhu and coworkers [196]. Here, DNA activity *in vivo* was reduced after 5-day exposure at day 8 postpartum, but not at day 70. Whether cadmium also induces changes in histone modifications is unknown to date.

Methylmercury (MeHg) is an environmental contaminant that can be present at relatively high levels in seafood. Recently, it has been shown for the first time that MeHg induces epigenetic alterations in mice. In 2007, Onishchenko and coworkers were able to demonstrate that male offspring of mice treated with MeHg during pregnancy showed a long-lasting effect on learning ability and a persistent predisposition to depressive behavior [197]. A follow-up study revealed neurotoxic effects of perinatal MeHg exposure in mice, resulting in long-lasting decreased expression levels of brain-derived neurotrophic factor (BDNF) [198]. BDNF is an important neurotrophin developmentally regulated in the prefrontal cortex, which

plays a significant role in neural survival, differentiation, neurite outgrowth, and synaptic plasticity [199, 200]. Further analysis revealed an increase in the silencing histone modification H3K27me3 as well as reduced H3K9/H3K14 acetylation at the *Bdnf* promoter IV in MeHg-exposed mice. In addition, the authors were able to correlate the reduced expression of BDNF mRNA to increased methylation levels of cytosine bases in CpG dinucleotides present in the *Bdnf* promoter IV by comparing MeHg-treated mice to untreated controls [198]. Studying the effects of MeHg on DNA methylation, Desaulniers *et al.* observed reduced DNMT1, 3A, and 3B mRNA levels in liver samples after *in utero* and postnatal exposure of rats [201]. In addition, the methylation level of CpG islands in the promoter of the tumor suppressor gene $p16^{Ink4a}$ was found decreased.

Aluminum

The research focus of Lukiw's lab has been the alteration of miRNA expression in human neural cells after exposure to ROS-generating neurotoxic metal sulfates [202–204]. The authors have demonstrated a significant upregulation of NF-κB-sensitive miR-146a and downregulation of complement factor H (CFH) in human neural cells upon aluminum sulfate treatment [203]. These data suggest that modulation of CFH through regulation by miR-146a may contribute to inflammatory responses on aluminum. In another study, they demonstrated an upregulation of the neural-specific or enriched miRNAs miR-9, miR-125b, and miR-128 upon exposure of primary human neural cells to iron or aluminum sulfate [204]. The same miRNAs were also upregulated in Alzheimer's diseased brain, thus suggesting a role of ROS-mediated toxicity of certain metals in progressive degenerative processes.

No information has been provided yet in the literature about miRNA regulation under exposure to metallotoxins other than arsenic, aluminum, or iron. However, several studies investigating the influence of metal-rich particulate matter in miRNA regulation provided evidence that metal components might significantly contribute to the effects exerted by particulate air pollutants (see below and Table 1). Altogether, one can state that metallotoxins should deserve increasing attention concerning their activities as epigenetic modulators. Aside low dose exposures to environmental contaminations, occupational exposures to such toxicants may constitute an unexpectedly high risk due to higher exposure doses over long periods of time.

Endocrine Disruptors and Reproductive Toxicants

As mentioned earlier, regulatory and differentiation processes in mammals are highly susceptible to alterations of gene expression patterns. Therefore, especially developing organisms are highly prone to misregulation by epigenetic modulators as well as endocrine disrupters. Some endocrine-disrupting chemicals (EDCs), aside having hormone-like activity by acting as agonists or antagonists in steroid

receptor signaling, may also exert an effect on the development through epigenetic modulation. The mammalian genome undergoes two cycles of global DNA demethylation/remethylation in mammalian germ cells and preimplantation embryos (reviewed in [4, 205, 206]). Additionally, the establishment of certain epigenetic and miRNA profiles is the basis for lineage specificity during development. There is evidence that exposure to different EDCs at critical time points may induce persistent epigenetic alterations.

Bisphenol A

The synthetic xenoestrogen bisphenol A (BPA) is widely used as a chemical in the manufacture of polycarbonate plastics which in turn are employed, for instance, in food and beverage packaging or in dental composites and sealants. Therefore, BPA raises attention not only as a general contaminant present in the environment, but is ingested on a daily basis by humans. A study reported by Calafat *et al.* in 2005 revealed measurable BPA levels in 95% of urinary samples from people in the United States [207]. Several *in vivo* studies have been carried out to investigate pre- and neonatal effects of BPA exposure in rats and mice. Ho and coworkers identified more than 50 candidate bands in a methylation-sensitive restriction fingerprint (MSRF) analysis of neonatally BPA-exposed rats [208]. Base pair resolution analysis of the candidate gene *Pde4d4* (phosphodiesterase type 4 variant 4 protein involved in cAMP pathway) by bisulfite sequencing identified a CpG island in the 5′ region of the gene, encompassing the transcriptional and translational start site. CpG dinucleotides at the 3′ end of the CpG island showed hypomethylation in BPA-treated mice when compared to control animals, increasing with animal aging. Methylation patterns correlated inversely with expression levels of *Pde4d4*, as analyzed by real-time PCR. Using the agouti mouse model (cf. above), Delinoy *et al.* demonstrated that *in utero* exposure leads to stable epigenetic alterations of the IAP element, resulting in a shift of coat color distribution in the offspring toward yellow [209]. Analysis of specific CpG sites in the promoter region of the A^{vy} IAP showed significant hypomethylation in BPA-exposed offspring when compared to control animals. Methylation levels in BPA-treated animals correlated highly in tissues derived from ecto-, meso-, and endoderm, as shown by analysis of the A^{vy} promoter methylation in brain, kidney, or liver, respectively. This suggests that the altered methylation pattern upon BPA exposure is established before germ layer differentiation and is stably maintained throughout differentiation and somatic proliferation. Hypomethylation in the presented study occurred as well at three distinct CpG sites of an additional IAP element inserted in the CDK5 activator binding protein (*Cabp*) gene, resulting in the $Cabp^{IAP}$ epiallele, indicating that BPA exposure leads to hypomethylation at multiple loci. Interestingly, maternal supplementation with methyl donors resulted in a coat color distribution of BPA-exposed offspring comparable to the untreated controls. CpG methylation at the A^{vy} promoter did not significantly differ from that observed in control animals. This effect could also be observed by supplementation with genistein, a compound which does

not serve as a methyl donor. Thus, epigenetic effects exerted by environmental chemicals seem to be part of a complex interplay of different factors, and nutrition is likely to be a major alterative factor (cf. above).

In 2008, Yaoi and colleagues presented a comprehensive study on the effects of BPA on DNA methylation using restriction landmark genomic scanning (RLGS) on DNA samples of the forebrain of *in utero* exposed mouse embryos [210]. Analyzing 2,500 loci in the mouse genome, they detected 1.9% of the spots to be differently pronounced in the exposed embryos when compared to the control. Analysis at two different developmental stages of control and BPA-treated individuals allowed in addition the identification of stage-dependent differences in DNA methylation. In total, they could correlate 13 loci to specific genes located on different chromosomes whose different methylation status was confirmed by quantitative PCR (qPCR). Twelve of these loci were located within a CpG island adjacent to the transcriptional start site of a functional gene. The data suggest that BPA-induced epigenetic changes appear indeed genome-wide and comprise hyper- as well as hypomethylation of specific loci.

A very recent study suggests that endocrine disrupters may exert their action in part by permanent epigenetic modification of responsive elements. Bromer *et al.* investigated effects after *in utero* BPA exposure in mice on the *Hoxa10* gene which controls uterine organogenesis [211]. Prenatal exposure to BPA led to an increased expression of *Hoxa10* and could be correlated to decreased DNA methylation at two CpG-rich regions, one located in the promoter region and encompassing the estrogen responsive element (ERE), and the second located intronically. Subsequent *in vitro* as well as *in vivo* experiments showed that CpG methylation in the ERE sequence significantly decreased estrogen receptor (ER) α-binding. An *in vitro* assay using the ER expressing breast carcinoma MCF-7 cell line finally showed that responsiveness to estradiol was significantly increased in cells carrying the hypomethylated promoter region of the *Hoxa10* gene [211]. In the same year, this group provided evidence that *in utero* exposure to BPA and diethylstilbestrol (DES; cf. below) increases the expression of EZH2 in mammary gland [212]. EZH2 is a catalytic subunit of polycomb repressive complex 2 (PRC2) with HMT activity and associated with breast cancer risk [213]. In addition, treatment of mammary carcinoma MCF-7 cells with BPA or DES also led to significant induction of EZH2 mRNA and protein expression. Histone H3K27me3 levels were increased in MCF-7 cells as well as in mammary gland of mice treated with both toxicants *in utero*. These results suggest that epigenetic changes upon BPA (or DES) exposure may contribute to an increased risk of breast neoplasia.

Analysis of the DNA methylation profiles of the ER promoter regions in adult testis of rats exposed to BPA for the first 5 days in life revealed a significant hypermethylation of ERα and ERβ [214]. Twofold induction of DNMT3A and 3B expression at mRNA and protein level was also observed in the same study. The authors suggested that the hypermethylation of the ER promoter could be a possible mechanism contributing to BPA-induced adverse effects on male fertility.

The examination of BPA effects on miRNA expression was carried out by Avissar-Whiting and coworkers [215]. The authors were able to show specific

changes in miRNA expression, in particular of miR-146a, after exposure of human placental cell lines to low concentrations of BPA for 6 days. miR-146a expression was strongly upregulated, and its overexpression in the cells reduced proliferation rate and made cells more sensitive to the DNA damaging agent bleomycin. Previously, miR-146a was shown to be upregulated in response to cellular stress induced by arsenic treatment or folate deficiency [124]. miR-146a implication in innate immune responses by regulating toll-like receptor and cytokine signaling was demonstrated by Taganov *et al.* [216]. Taking together, miR-146a may play a significant role in the cellular responses to environmental stress and inflammation.

Diethylstilbestrol

The synthetic estrogenic chemical DES provides another example of an EDC that has been shown to exert epigenetic modulator activity. In earlier times, DES was used for treatment of pregnant women to prevent miscarriage and other pregnancy-related complications. In the early 1970s, first reports linked DES to reproductive tract cancer in female offspring and following studies expanded information on DES acting as an estrogenic toxicant (reviewed in [217]). Even though DES is not implemented anymore as pharmacological agent, observations of transgenerational effects hold the informative value high concerning its epigenetic action. One of the first reports concerning epigenetic alterations after DES treatment was based on the observation of DNA hypomethylation in estrogen-induced hamster kidney tumors from Lu *et al.* [218]. This effect is most likely due to a global hypomethylation that is more generally observed in malignant cells, rather than anything specific for DES. A DES-specific demethylation, which can be early observed after DES treatment and which is maintained even in developed tumors, was reported in mice in the late 1990s by Li *et al.* [219]. Here, investigation of the methylation status of CpG dinucleotides in the promoter region of the lactoferrin gene (an estrogen-regulated member of an iron-binding glycoprotein family expressed in various tissues, including mammary gland and reproductive organs) revealed significant demethylation of a single CpG residue after neonatal treatment with DES when compared to control animals and was still observed in mature uteri of mice. This demethylation effect was found to be dependent on endogenous ovarian hormones in adulthood. The same group also reported on hypomethylation of six CpG loci in exon 4 of the proto-oncogene *c-fos* in the uteri of mice that were exposed for 5 days to DES after birth, resulting in persistent overexpression of this immediate early gene.

Work from Tang and coworkers identified 14 differentially methylated loci in the mouse genome after neonatal exposure to DES by MSRF that unequivocally mapped to CpG islands in the proximal promoter region, exon 1, and/or intron 1 of protein-coding genes [220]. Six of them showed hypomethylation, while eight appeared hypermethylated in mice treated with DES. Closer analysis of the promoter of *Nsbp1* (nucleosomal binding protein 1), whose gene product is involved in chromatin remodeling, revealed a significant increase in methylation in control animals throughout maturation. The hypermethylated state remained further in life.

In contrast, animals exposed right after birth to DES in different concentrations showed a hypomethylated state of the *Nsbp1* promoter, which also remained throughout life. Methylation status correlated inversely with expression levels as analyzed by qPCR. Since these methylation differences between treated and control animals could not be observed in ovariectomized animals, as in the study analyzing the lactoferrin gene, the life reprogramming of *Nsbp1* gene expression through methylation, which is disturbed by neonatal exposure to DES, seems to be dependent on normal adult ovarian steroid expression. Two other studies from Mori's lab on the effects of neonatal DES exposure on male and female mice revealed differential expression of DNMT1, 3A, and 3B at three different time points when compared to control animals (even though not all DNMTs were differentially expressed at the same time points) [221, 222]. By RLGS, they were able to show different mismethylated CpG loci in the epididymis as well as in the uteri of treated animals when compared to the controls.

As mentioned above, DES and BPA are capable of inducing the expression of the methyltransferase EZH2 and—as a consequence—increasing H3K27 methylation levels in mammary glands and carcinoma cells upon *in utero* or *in vitro* exposure [212]. Conversely, Bredfeldt *et al*. were able to show that DES induces phosphorylation of EZH2 in MCF-7 cells or in the developing uterus through activation of a rapid/nongenomic ER-mediated PI3K/AKT signaling pathway [223]. The phosphorylation of EZH2 reduces its methyltransferase activity, thereby resulting in the reduction of H3K27 methylation levels [223, 224].

Recently, Hsu and colleagues have analyzed epigenetic effects of DES using a progenitor-containing mammospheres *in vitro* model [225]. These progenitors are self-renewal cells that can be differentiated into different types of breast epithelial cells *in vitro*. Here, expression levels of 9% of tested miRNAs were found altered in epithelial offspring from mammospheres exposed to DES [225]. Expression of miR-9-3, a potential regulator of the TP53-related apoptotic pathway, was strongly downregulated in epithelial cells preexposed to DES. An epigenetic analysis of the corresponding genome locus revealed several repressive marks, including polycomb EZH2-mediated chromatin modifications, i.e., H3K27me3 and H3K9me2. Moreover, an association of DNMT1 with the *miR-9-3* locus and—as a result of its action—an aberrant increase in DNA methylation of promoter CpG islands was observed after DES preexposure of epithelial cells. Combined treatment of the cells with the demethylation agent 5-aza-2′-deoxycytidine and the HDAC inhibitor trichostatin A restored the expression of miR-9-3. Thus, epigenetic repression of the tumor suppressive *miR-9-3* gene promotes proliferation of breast epithelial cells that makes this gene an important signal of early breast cancer development.

Vinclozolin and Methoxychlor

Based on work published in 2005 from the Skinner laboratory, the commonly used fungicide vinclozolin started to raise increased attention. The metabolism of vinclozolin leads to products which exert antiandrogenic effects [226]. The Skinner lab

was able to show that intraperitoneal application of vinclozolin to gestating Sprague–Dawley rats leads to an increase in spermatogenetic cell apoptosis, reduced epididymal sperm counts, and sperm motility up to the F4 generation and that the phenotype is transmitted through the male germ line [227]. The observed phenotypes were proposed to be based on epigenetic changes since genetic mutation would not result in such a high frequency and analysis of the methylation pattern in testes after exposure of F344 rats to vinclozolin resulted in the identification of multiple loci showing mismethylation up to the F3 generation [227]. In an attempt to corroborate a transgenerational epigenetic effect, the same group published an MSRF analysis of the F3 generation of vinclozolin-treated F0 animals and identified 25 mismethylated loci which were sequenced and further characterized for their distinct methylation patterns [228]. However, Schneider *et al.* treated gestating Wistar rats orally with vinclozolin but failed to reproduce the transgenerational effect that had been published 3 years before [227, 229]. It has to be mentioned that even though additional studies have been published from the Skinner laboratory that deal with the so-stated "transgenerational epigenetic effects of the endocrine disrupter vinclozolin" [230–232], the study published on methylation analysis of the F3 generation in 2006 has been retracted in 2009. One year later, another study has been published by Skinner and coworkers describing the transgenerational effects of vinclozolin on the sperm epigenome [233]. The authors were able to determine 16 different regions with mismethylated promoter profiles in sperm cells of F3 generation rats whose F0 generation mother had been exposed to vinclozolin. Thus, the authors concluded that endocrine-disrupting agents such as vinclozolin are capable of changing the epigenome of sperm cells transgenerationally. The putative transgenerational epigenetic effects of EDCs have recently been summarized in a review article [234]. In summary, the effects observed after vinclozolin exposure remain enigmatic, and the intriguing data described definitely deserve further work to finally clarify the mechanisms underlying the putative transgenerationally and epigenetically mediated effects of certain compounds such as vinclozolin.

In 2009, Zama and Uzumcu reported on the effects of perinatal exposure of rodents to the pesticide and endocrine disruptor methoxychlor [235]. Methoxychlor has been shown to lead to similar phenotypes after perinatal exposure in rats [236], as have been observed after vinclozolin treatment [237]. The authors report on hypermethylation of the ERβ promoter region as well as on the identification of 10 genes in ovaries of exposed rats, as determined by arbitrarily primed (AP-)PCR. By analyzing mRNA expression levels of the different DNMTs, they showed a stimulation of DNMT3B expression, whereas DNMT3A and DNMT3L remained unaffected [235]. Further, the analysis of possible transgenerational effects of these compounds on imprinted genes revealed that treatment of pregnant mice leads to an alteration of both paternally (*H19*, *Gtl2*) and maternally (*Peg1*, *Snrpn*, *Peg3*) imprinted genes in the male gametes, but not in somatic cells of F1 offspring [230, 238]. From these data, the authors concluded that imprinting alterations in sperm cells may contribute to the understanding of previously reported effects of EDCs on male spermatogenesis and fertility rates.

While EDCs have been in the center of attention for a long time, investigating the putative epigenetic activities of these substances is relatively new. Therefore, one can assume that expanding research will help unraveling some of the mechanisms of how EDCs act on mammalian organisms to exert their effects.

Complex Particulate Air Pollutants

Particulate Matter

Particulate air pollutants mainly originate from human activity such as combustion of fossil fuel, biomass, waste, and other industrial processes. Increasing numbers of epidemiological, clinical, and *in vivo* studies have been carried out in order to investigate the possible health effects of inhaled particulate matter (PM) pollutants. Enhanced concentrations of PM in the environmental atmosphere have been associated with increased mortality and morbidity in the general population resulting from cardiovascular and respiratory diseases (reviewed in [239–241]). A number of studies have shown that inhalation of PM is associated with alteration in gene expression, activation of inflammatory pathways, and production of ROS (reviewed in [242, 243]); however, the precise molecular mechanisms remain unclear. There is increasing evidence that epigenetic effects contribute to the health risks exerted by PM exposure. Epidemiological studies are helpful to correlate exposure to disease, but elucidating responsible mechanisms leading into a state of disease remains demanding. In particular, the term "particulate matter" comprises a large variety of particles and substances that altogether build up air pollutants that are ubiquitously present and that may contribute to the development of certain diseases.

A study from Hou and coworkers from the late 1990s published the analysis of autopsied lungs of 47 patients who had died of pulmonary adenocarcinoma, with respect to a correlation of black dust matter and hypermethylation of the tumor suppressor gene $p16^{Ink4a}$ [244]. They found a high correlation of $p16^{Ink4a}$ methylation (and reduced $p16^{INK4A}$ protein expression) to tumors with severe anthracosis and suggested that the level of background anthracosis is associated with $p16^{Ink4a}$ silencing in pulmonary adenocarcinogenesis. $p16^{Ink4a}$ promoter methylation was also observed in lung tumors developed upon exposure of rats to particulate carbon black and diesel exhaust [245]. In a further study on peripheral blood leukocytes from steel workers exposed to PM, higher methylation levels of the tumor suppressor *Apc* gene promoter were observed [246]. By contrast, the promoters of two other tumor suppressor genes, *p53* and *Rassf1a*, were found hypomethylated. In 2009, Baccarelli *et al*. demonstrated a significant decrease in blood cell DNA methylation of Alu elements and Line-1 (long interspersed nuclear element-1) after exposure to high levels of traffic particles [247]. The same group showed slightly reduced methylation levels of the aforementioned repetitive elements in steel production plant workers [248], reinforcing the observed effects of PM exposure on hypomethylation of repetitive DNA elements. In addition, it was demonstrated that

promoter methylation of the inducible nitric oxide synthase gene (*iNos*) was reduced after 3-day exposure to PM. This observation was in line with *in vitro* studies that have shown that ROS, which are most likely one of the main cellular stressors generated by PM exposure [249], may produce genomic hypomethylation [250] and that *iNos* expression is elevated in the presence of ROS [251].

Analysis of histone modifications in peripheral blood leukocytes from steel workers exposed to metal-rich PM revealed a correlation between air levels of nickel and the increase of H3K4me2 levels [252]. Further, cumulative exposures to either nickel- or arsenic-rich PM were positively correlated to both H3K4me2 levels and H3K9 acetylation, suggesting histone modifications as novel epigenetic mechanism underlying the adverse effects of inhalable nickel and arsenic containing matter [252]. In parallel, the same group has investigated the effects of exposure to PM and metallic PM components (chromium, lead, cadmium, arsenic, nickel, manganese) on miRNA expression [253]. Three miRNAs, i.e., miR-222, miR-21, and miR-146a, were in the focus of this study. These miRNAs are believed to regulate the expression of the genes that participate in response to oxidative stress and inflammation [115, 254, 255]. miRNA expression was analyzed in blood leukocytes after 3 days of work. A significant increase in miR-222 and miR-21 expression in postexposure samples has been observed, while miR-146a expression was not significantly changed. miR-222 overexpression was positively correlated with lead exposure, while miR-21 expression was associated with the induction of 8-hydroxy-2′-deoxyguanosine (8-OH-dG), but not with metal components, confirming the suggestion that miR-21 expression is a part of responses to oxidative stress [115]. The authors concluded that miRNA changes may represent an additional mechanism of cellular response to PM and its metal components. However, further studies are required to investigate which cellular pathways are particularly affected by the observed miRNA alterations induced through PM exposure.

Supporting these observations, Jardim and coworkers were able to show alterations in miRNA expression profiles in human airway epithelial cells in response to exposure to diesel exhaust particles *in vitro* [256]. In this study, the expression of more than 50% of tested miRNAs has been found affected in comparison to the control. The cohort of deregulated miRNAs included in particular those which are thought to be associated with inflammatory responses and tumorigenesis.

The cardiovascular toxicity of PM-associated metals produced by fossil fuel combustion was analyzed by Farraj *et al.* [257]. Spontaneously hypertensive rats, an animal model commonly used for the study of cardiovascular diseases, were exposed for 4 h to iron-, nickel-, and vanadium-rich PM. Aside electrocardiogram (ECG) affection and pulmonary inflammation, alterations in the expression of myocardial-specific miRNAs were observed after exposure to high concentrations of PM. At lower concentrations, lesser effects could be observed on inflammation and miRNA levels, while some changes in the ECG were still present. The cardiac-specific miRNAs, i.e., miR-1 and miR-133, which have been shown to modulate heart development and inhibit cardiac hypertrophy, were strongly affected by PM, but their known target genes, *kir2.1* and *connexion-43*, remained unaffected. It is thus not clear so far how downregulation of these miRNAs may alter cardiac functions. Further, miR-21,

miR-24, and miR-29, which were shown to be implicated in heart failure, cardiac hypertrophy, and fibrosis (reviewed in [258, 259]), were also downregulated after exposure to high concentrations of PM. The expression levels of several other miRNAs with unknown roles in cardiovascular functions were reduced by PM as well. Some of them (miR-26, miR-99, miR-122, miR-125, miR-145, miR-146, and miR-191) were also downregulated in rat lungs after cigarette smoke exposure (cf. below). Further investigations are necessary to elucidate the exact role of miRNAs and general epigenetic effects in PM-mediated adversity.

Cigarette Smoke

Cigarette smoke represents an example of PM exposure that has been the subject of many studies in the past, due to a strong correlation between smoking and the development of lung cancer and a variety of chronic degenerative diseases. Lung cancer belongs to the most lethal malignancies worldwide. As mentioned earlier, aberrant DNA methylation is ubiquitously observed in malignant tissues. Especially for lung cancer, several studies have been published on molecular events in carcinogenesis and also on aberrant promoter methylation (reviewed in [260, 261]). The observation that aberrant promoter methylations of the $p16^{Ink4a}$ tumor suppressor gene in squamous cell carcinoma (SCC) and of *Mgmt* in non-small-cell lung cancer (NSCLC) are early and very frequent events made promoter methylation analysis of such genes, as well as of other tumor-related genes, a promising tool for biomarker analysis at early stages of cancer [262–264].

In 2002, Belinsky and colleagues presented a study investigating the methylation status of $p16^{Ink4a}$, *Mgmt*, *Dapk*, and *Rassf1a* in bronchial epithelial cells as well as in sputum of cases and control persons who were current or former smokers [265]. The results indicate that promoter hypermethylation of the $p16^{Ink4a}$ gene and—less frequently—of *Dapk* occurs with high incidence in the bronchial epithelium of lung cancer cases and cancer-free controls who smoked and that these epigenetic changes persist even after cessation of smoking [265]. In contrast, *Rassf1a* promoter methylation was not observed. The absence of significant differences in promoter hypermethylation of $p16^{Ink4a}$ and *Dapk* between cases and controls suggests that promoter hypermethylation of the aforementioned genes is rather an early event, permissive for acquisition of additional changes that might lead into a state of disease. Long-term exposure of human small airway epithelial cells and CDK4/hTERT-immortalized human bronchial epithelial cells to cigarette smoke condensate revealed changes in DNMT expression and histone modifications (i.e., reduction of H4K16 acetylation and H4K20me3 levels, as well as increases in H3K27me3 levels) [266]. In contrast to previous studies [265], under these conditions, hypermethylation of the *Rassf1a* promoter has been observed, and repetitive DNA sequences, such as *Line-1*, *D4z4*, and *Nbl2*, were found hypomethylated. Moreover, progressive genome hypomethylation and regional DNA hypermethylation led to the formation of colonies in soft agar, indicating that cigarette smoke condensate has the potential to direct the epigenome of human bronchial epithelial cells toward a cancer-associated phenotype [266].

The lack of appropriate animal models resulting in statistically significant tumor formation upon cigarette smoke exposure rather made it difficult to characterize the influence of cigarette smoke on the epigenome during development of malignancies. The group of Belinsky reported in recent years on studies using B6C3F1 mice for exposure to mainstream cigarette smoke and analyzed the methylation status of selected genes in developed lung tumors. In a study from 2004, they report on the hypermethylation of the *Dapk* promoter in tumors developed upon 30 weeks of exposure to cigarette smoke [267]. The prevalence of methylation in tumors was identical in those developed after treatment of mice with the tobacco carcinogens 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and vinyl carbamate.

Several *in vivo* studies were carried out by Izzotti and colleagues to investigate the role of cigarette smoke on miRNA expression patterns [268–271]. Exposure of rats to environmental cigarette smoke (side and mainstream smoke) for 28 days causes significant downregulation of miRNA expression in rat lungs. Notably, miRNA alterations occur in this case as an early response on the exposure of lung tissue before the onset of malignancy. The most remarkably downregulated miRNAs belonged to the families which were shown to regulate stress response, inflammation response, apoptosis, proliferation, and angiogenesis, thereby playing a significant role in carcinogenesis [270]. Previous studies of gene and protein expression profiles revealed a panel of genes and proteins being upregulated in the same tissue upon cigarette smoke exposure [272] and that were consistent with the role of miRNAs in translation repression. One miRNA, miR-294, was upregulated in lungs of rats exposed to cigarette smoke. The predicted targets of this miRNA are transcriptional repressors, such as zinc-finger protein (ZNF) 697 and AT-rich interactive domain 4A. Thus, increased silencing of transcriptional repressors by miR-294 may lead to a global increase of gene transcription.

Oral treatment of cigarette smoke-exposed rats with various chemopreventive agents (*N*-acetylcysteine, oltipraz, indole-3-carbinol, 5,6-benzoflavone, and phenethyl isothiocyanate) led to attenuation of cigarette smoke-mediated effects on miRNA expression, indicating a potential protective effect of those drugs on miRNA alterations induced by environmental noxious agents with carcinogenic potency [268]. Similar results were observed after exposure of neonatal mice to cigarette smoke and subsequent treatment with phenethyl isothiocyanate or budesonide, but with some adverse effects in liver [271]. In humans, the effects of smoking on the "miRnome" and transcriptome in bronchial airway epithelium were analyzed by Schembri *et al.* [273]. Contrarious changes were observed in the expression levels of a number of miRNAs and their target mRNAs in current smokers in comparison to nonsmokers [273]. In particular, miR-218 was strongly affected by cigarette smoke. Ectopic overexpression or knockdown of this miRNA in bronchial epithelial cells led to a corresponding decrease or increase in the expression of miR-218 predicted targets, thus supporting the idea that miRNAs may modulate gene expression in response to environmental toxins and, therefore, could serve as indicators of smoking-induced diseases.

The tobacco-specific nitrosamine NNK (cf. above) has been well characterized as a potent lung carcinogen [274]. Kalscheuer and colleagues have analyzed the

miRNA expression profiles in the lungs of male F344 rats after treatment with NNK for 20 weeks [275]. A range of miRNAs, such as miR-34, miR-101, miR-126, and miR-199, were significantly downregulated. This pattern correlated well with miRNA profiles detected in human cancer tissue [276]. Moreover, CYP2A3, an identified target enzyme of miR-126 and known metabolic catalyst of NNK, was upregulated in response to NNK. Thus, the interaction of miR-126 and CYP2A3 could be a potential mechanism contributing to NNK carcinogenicity.

Pharmacological Agents

Valproic Acid and Lithium

Mood stabilizers such as lithium and valproic acid (VPA) alter a wide range of intracellular signaling processes in the brain regions involved in mood regulation and are used as drugs for treatment of bipolar disorders. VPA is known to inhibit proliferation and to promote apoptosis and differentiation; therefore, this compound is currently under consideration for cancer treatment [277]. The main but not exclusive mechanism of regulation of gene expression by VPA is mediated through HDAC inhibition [278]. Moreover, VPA has been identified as a teratogenic agent, the embryotoxic effects of which have been ascribed to malformations of the heart and neural tube closure defects [279]. HDAC inhibition has been suggested to be a major cause of VPA teratogenicity [280].

Despite this knowledge, the molecular mechanisms of VPA and lithium action remain not completely understood. Since miRNAs have been shown to play a critical role in regulation of neurogenesis, neurite outgrowth, synaptogenesis, and synaptic plasticity (reviewed in [281]), two labs have hypothesized that VPA and lithium may alter the expression of miRNAs and their target genes, which might be involved in therapeutic effects of these drugs [282, 283]. Zhou and colleagues have demonstrated changes in the expression of hippocampal miRNAs after chronic treatment of rats with VPA and lithium [282]. Several potential target genes of deregulated miRNAs, which are involved in neurogenesis, neurite outgrowth, signaling of PTEN, ERK, and WNT/β-catenin pathways, were upregulated after treatment. To verify these *in vivo* results, the authors incubated primary hippocampal neurons with lithium or VPA and could demonstrate that expression of miR-34a and its putative target gene, i.e., metabotropic glutamate receptor 7 (*Grm7*), was reciprocally changed after treatment, confirming that miR-34a regulates GRM7 levels and contributes to the effects of VPA and lithium on GRM7 expression [282]. Chen and coworkers addressed the same question but used human lymphoblastoid cell lines as the test system [283]. Four miRNAs (miR-34a, miR-152, miR-155, and miR-221) were significantly upregulated upon lithium treatment; however, the expression of miR-34a and miR-221 was changed in opposite direction as was shown for rat hippocampus [282], thus demonstrating a tissue-specific response to lithium treatment.

Paracetamol

Fukushima *et al*. reported on the alterations of miRNA expression in liver following treatment of rats with known hepatotoxicants, i.e., acetaminophen (APAP, paracetamol) and carbon tetrachloride (CCl_4) [284]. APAP is broadly used as analgesic and antipyretic, but excessive use may cause liver injury [285]. CCl_4 was widely used in fire extinguishers and as a cleaning agent. Chronic exposure to CCl_4 causes liver damage and oxidative stress and was shown to exert carcinogenic potency [286, 287]. Among the pattern of miRNAs affected by both hepatotoxicants, miR-298 and miR-370 were of particular interest because they are predicted to regulate oxidative stress-related genes, and APAP and CCl_4 are known to upregulate these genes. These studies also demonstrated that the alteration of miRNA expression occurs in early stages of hepatotoxicity, prior to the onset of cellular necrosis. Association of miRNA expression changes in liver and plasma with APAP-induced liver injury was also shown by Wang *et al*. [288]. The authors suggested that specific circulating miRNA (such as miR-122 and miR-192) can be used as sensitive blood-based biomarkers for liver injury.

Tamoxifen

Tamoxifen, an antagonist of the estrogen receptor (ER), is widely used in chemotherapy of breast cancer. However, metabolically activated tamoxifen was shown to be a potential hepatocarcinogen in rats as well, acting through the formation of tamoxifen-DNA adducts [289]. Global epigenetic changes at early stages of tamoxifen-induced tumorigenesis were observed by Tryndyak *et al*. in F344 rat liver that included the decrease of cytosine DNA methylation, reduction of histone H4K20me3 levels, and alteration of *de novo* DNA methylation [290]. By contrast, no epigenetic changes could be observed in nontarget tissues (e.g., pancreas and spleen). One year later, the same group investigated miRNA expression profiles in F344 female rats after treatment with tamoxifen for 24 weeks [291]. The authors have demonstrated upregulation of known oncogenic miRNAs (i.e., miR-34, miR-17-92 cluster, and miR-106a) in liver tissue prior to tumor formation. Reciprocal expression patterns of miRNAs and their confirmed targets, which included cell cycle regulators, chromatin modifiers, and expression regulators, were observed. For instance, retinoblastoma (RB) 1 protein expression (a target of miR-106a) was reduced by 56%. The RB1 family of tumor suppressors plays a significant role in heterochromatin formation and in the regulation of gene expression by recruiting chromatin modifiers to promoter regions [292]. Alteration in the expression of miR-106a and its target RB1 prior to tumor formation, along with substantial reduction of histone methylation, provides again evidence for the interplay of epigenetically active enzymes and miRNAs.

Supporting these results, recently, Pogribny and coworkers have demonstrated epigenetic alterations upon long-term treatment of rats with another powerful carcinogen, i.e., 2-acetylaminofluorene (2-AAF) [293]. Similar as with tamoxifen treatment, alteration of miRNA expression levels and changes in epigenetic marks in promoter

regions of tumor suppressor genes were observed in preneoplastic livers of rats exposed to 2-AAF [293]. In particular, a global loss of histone H4K20me3 but increases in H3K27me3 and H3K9me3 levels at the promoter regions of the tumor suppressor genes *Rassf1a*, $p16^{Ink4a}$, *Socs1*, *Cdh1*, and *Cx26* were observed. miR-31 was found significantly upregulated and associated to the downregulation of CEBPA protein, a known transcriptional inhibitor of the hepatocarcinoma marker gene *Gstp* (glutathione *S*-transferase p). These results demonstrate the crucial role and the importance of epigenetic and miRNA alterations in initial stages of hepatocarcinogenesis induced by long-term exposures to chemical carcinogens [294].

Early downregulation of tumor suppressor miRNAs (i.e., miR-34a, miR-127, miR-200b, miR-16a) was observed in rat liver carcinogenesis induced by a methyl-deficient diet. The expression levels of these miRNAs were invariable at later stages of tumor formation and in fully developed tumors [113]. The oncogenes *Bcl2*, *Bcl6*, and *E2f3* have been confirmed as targets for downregulated miRNAs and were upregulated under methyl-deficient conditions. This finding further demonstrated that the imbalance of miRNAs and their targets may play a crucial role in carcinogenesis.

Peroxisome Proliferators

The induction of peroxisome proliferation-activated receptor α (PPARα) by PPARα agonists (e.g., lipid-lowering drugs such as fenofibrate, gemfibrozil, clofibrate) has been well characterized as a mechanism of hepatocarcinogenesis in rodents [295]. However, the precise molecular mechanism of gene expression regulation by PPARα activation remains ambiguous. Therefore, Shah and coworkers experimentally addressed the question if miRNAs could be involved in peroxisome proliferator-induced carcinogenesis [296]. Liver miRNA expression profiles were analyzed after 2-week treatment of mice with the PPARα agonist Wy-14,643. The expression levels of 15 miRNAs were found significantly increased, while 12 miRNA species were downregulated. In particular, the hepatic level of tumor suppressor miRNA let-7c was reduced, while the expression of its target oncogene, *c-Myc*, was elevated following 4 h, 2 weeks, or 11 months of treatment with Wy-14,643. As a consequence, the transcription of the oncogenic miR-17-92 cluster was strongly induced. In experiments where let-7c was overexpressed, downregulation of both c-MYC and miR-17, as well as growth suppression of Hepa-1 cells, could be observed. These findings suggest a critical role of the tumor suppressive miRNA let-7c in response to peroxisome proliferator-induced liver cell proliferation and tumorigenesis [296].

Other Drugs

Saito and coworkers were able to show a strong upregulation of different miRNAs in T24 human bladder cancer cells after treatment with the well-known

chromatin-modifying drugs 5-aza-2′-deoxycytidine and 4-phenylbutyric acid [80]. Out of 313 analyzed human miRNAs, they identified a total of 17 miRNAs that were upregulated more than threefold after combinatorial treatment with these two drugs. The strongest effect was observed for miR-127 (50-fold upregulation) that may downregulate the *Bcl6* oncogene (cf. above). Toyota and colleagues have shown the same effect of 5-aza-2′-deoxycytidine on miR-34b/c expression in colorectal cancer [81]. Since some miRNAs have been classified as tumor suppressors (e.g., miR-127, miR-34, miR-15, miR-16) and others as oncogenes (e.g., miR-21, miR-155, miR-372) [44, 95, 297], the alteration of epigenetic control of those genes may contribute to a malignant phenotype. The demonstration that epigenetic drugs may activate tumor suppressive miRNAs helps to understand the drug action mechanisms and opens new possibilities in cancer treatment.

A classical antimetabolite, 5-fluorouracil (5-FU), which is widely used as anticancer drug, was shown to alter the expression of several miRNAs in colon cancer cells, including antiapoptotic miR-21 and miR-200b [298]. miR-200b has been found upregulated in various tumors but was downregulated in colon cancer cells after 5-FU treatment. By contrast, the expression of miR-21 was induced. Since miR-21 is a known oncogene that is commonly overexpressed in malignant tissues, the induction of miR-21 expression by 5-FU has been judged as cellular response to this strongly toxic agent [298].

Other Compounds

Hexogen

Zhang and Pan evaluated changes in miRNA profiles in mouse liver and brain under exposure to RDX (*Research Department Explosive*/*Royal Demolition Explosive*) [299]. RDX, also known as hexahydro-1,3,5-trinitro-1,3,5- triazine, hexogen, or cyclonite, is a highly explosive environmental pollutant resulting from military and some industrial activities. RDX exposure has been associated with neuro- and immunotoxicity and an increased risk of cancer. It has been shown that feeding of mice with 5 mg/kg RDX for 28 days led to alterations in the expression of 113 miRNAs in liver and brain, many of which were related to toxicant-metabolizing enzymes, neurotoxicity, and different cancer types [299]. The authors demonstrated tissue-specific alterations of miRNA expression in response to RDX with more pronounced regulation of brain miRNAs than that of the liver. The tissue-specific variations supported the idea that miRNAs play different roles in different tissues in response to xenobiotics. Moreover, upregulation of oncogenic miRNAs and downregulation of tumor suppressing miRNAs, including let-7, miR-17-92, miR-10b, miR-15, miR-16, miR-26, and miR-181, were observed. The significant alteration of miRNA expression in the brain upon RDX exposure may clarify so far unknown molecular mechanisms of RDX-induced neurotoxicity. Thus, miR-206, miR-195, and miR-30a were upregulated

in the brain by 26.5-, 3.6-, and 2.6-fold, respectively [299]. Each of the three miRNAs was predicted to target the brain-derived neurotrophic factor (BDNF) [199, 200]. *Bdnf* downregulation by miR-195 and miR-30a has been experimentally confirmed by Mellios and colleagues [300]. BDNF was already shown to be associated with the neurotoxicity of environmental toxicants (cf. above and [301, 302]); therefore, the RDX-induced increases in the expression of *Bdnf*-specific miRNAs may contribute to the neurotoxicity of RDX.

Ethanol

Ethanol is a powerful toxin that exerts diverse health effects, including the promotion of neurodegenerative, hepatic, and cardiovascular diseases. Several groups have addressed the question whether ethanol's health effects could be modulated by miRNAs. The role of miRNAs in ethanol toxicity has been recently reviewed by Miranda and colleagues [303]. Briefly, it was demonstrated that miRNAs may contribute to the molecular mechanisms of ethanol tolerance development, an initial step of addiction [304]. So, miR-9 was found upregulated in primary mice neurons in response to ethanol exposure, while its putative target, an ethanol-sensitive isoform of the α-subunit of BK (big potassium) ion channel, was repressed. The ethanol-induced miR-9 binding to its target shifts the ratio of ethanol-sensitive/ethanol-tolerant isoforms toward tolerance. In addition, the authors demonstrated that ethanol-induced upregulation of miR-9 may repress the expression of at least 10 other genes that are involved in neurotransmitter release, synaptic plasticity, and circadian rhythm [304]. Implication of miRNAs in the development of ethanol-induced gastrointestinal diseases, such as alcoholic liver disease (ALD) and gut leakiness, was demonstrated by Tang *et al*. [305]. The authors were able to show that miR-212 is highly expressed in intestinal epithelial cells and was induced upon ethanol exposure, while its target, zonula occludens 1 (ZO1), was downregulated. ZO1 is a key tight junction protein that contributes to the intestinal barrier. As a consequence of ZO1 repression, ethanol-induced disruption of monolayer integrity and increased permeability of epithelial cells were observed. These findings were supported by colon biopsies from ALD patients, where miR-212 was overexpressed and ZO1 was downregulated [305], suggesting a novel mechanism of ethanol-induced gut leakiness.

Prenatal exposure to ethanol may lead to the development of fetal alcohol syndrome that is characterized by mental retardation, behavioral problems, poor growth, craniofacial, cardiovascular, and skeletal defects. Significant suppression of miR-21, miR-9, and miR-335 was observed after exposure of mouse fetal cerebral cortex-derived neuroepithelial cells *ex vivo* to ethanol in a dose simulating daily consumed amounts of alcohol by alcoholics [306]. Lower ethanol concentrations, corresponding to social drinking, induced miR-335 expression. Previously, the same group has shown the induction of proliferation of neuroepithelial cells upon exposure to ethanol [307]. The demonstration that antiapoptotic miR-21 and proapoptotic miR-335 function as antagonists suggests the possible mechanism of ethanol-sensitive regulation of neuroepithelial survival and growth by miRNAs [306]. Wang and colleagues found

ethanol-sensitive miR-10a upregulated in fetal mouse brain upon prenatal ethanol exposure, while the *Hoxa1* gene was downregulated [308]. HOXA1, a transcription factor regulating morphogenesis and cell differentiation during development, is a predicted target for miR-10. Treatment with folic acid prevented ethanol-induced teratogenesis among others by induction of HOXA1 and reduction of miR-10 expression. This finding may contribute to the understanding of the pathogenesis of fetal alcohol syndrome and the role of miRNAs in ethanol teratology [308].

Dioxins and Benzopyrene

Dioxins (polychlorinated dibenzo-*p*-dioxins) have long been characterized as extremely toxic and globally distributed environmental pollutants exerting teratogenic, mutagenic, carcinogenic, and immunosuppressive effects in humans [309]. These compounds are known agonists of the AHR that are capable of altering the transcription level of numerous genes, but specific targets whose deregulation would contribute a major factor to dioxin-mediated toxicity remain largely ambiguous. Moffat and colleagues have suggested that miRNAs could be involved in the downregulation of certain target transcripts [310]. However, they have observed only minor alterations in the expression levels of a small panel of hepatic miRNAs after treatment of rats with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most potent dioxin congener [310]. The authors suggested that the tiny changes in miRNA expression are a secondary effect of TCDD toxicity and that it is unlikely that miRNAs contribute to a significant extent to TCDD-mediated downregulation of mRNA levels. However, recently miR-191, a potential inducer of hepatocellular carcinoma development, was shown to be transcriptionally upregulated by TCDD through activation of the AHR signaling pathway [311]. These results suggest a possible role of this oncogenic miRNA in TCDD-induced toxicity and carcinogenesis.

Benzo[*a*]pyrene (BP) belongs to the group of environmental polycyclic aromatic hydrocarbons (PAHs) and is another well-known AHR agonist exerting strong carcinogenicity in a range of different organs in rodent tumor models (see chapter on *Exposure to Polycyclic Aromatic Hydrocarbons: Bulky DNA Adducts and Cellular Responses*, authored by Henkler *et al.*). Recently, it has been shown that BP was unable to alter miRNA expression in adult mouse liver *in vivo* [312]. It just exhibited a minimal direct effect on hepatic miRNA levels after short-time exposure (4 days). However, long-term exposure to the most important carcinogenic metabolite of BP, i.e., *anti*-BP-7,8-diol-9,10-epoxide (BPDE), revealed strong alterations in the expression of 54 miRNAs in malignantly transformed human bronchial epithelial cells [313]. In particular, miR-10a was significantly downregulated in the transformed cells, while its putative target gene, *Hoxa1*, was strongly upregulated. Since HOXA1 and miR-10a were shown to be involved in the development of megakaryocytopoiesis and adult myeloid leukemia [314], the decrease of miR-10a expression may contribute to the BPDE-mediated malignant transformation of bronchial epithelial cells. Moreover, the following oncogenic miRNAs were found upregulated in BPDE-transformed cells:

miR-17-92 cluster, miR-21, miR-27a/b, and miR-141. Changes in the miRNA profiles presented in this study may therefore contribute to the malignant transformation of cells exposed to BPDE.

Conclusion

The current knowledge about the epigenetic and miRNA responses on drugs, chemicals, and environmental toxicants has been reviewed in this chapter. Without any doubt, further research in the epigenetic field, including the expanding world of miRNAs, will provide new insights into mechanisms of cellular responses to toxic agents. In particular, it is expected that this direction will emphasize the role of miRNAs in toxicology. Considering the link between miRNA expression and diverse pathological and toxicological processes, miRNA profiling—along with transcriptome and proteome analyses—will serve as an excellent tool for the understanding of toxicity pathways, compound-mediated aberrant physiology and diseases, as well as for the development of new biomarkers and pharmaceuticals. To this end, it becomes absolutely necessary, however, to evaluate and understand more precisely the biology and physiology of epigenetic and miRNA alterations. The interrelation between epigenetic changes induced in cells, tissues, and organs of humans exposed to drugs and environmental toxicants and the possible adverse health outcome is something that requires much more attention and that certainly needs to be comprehensively addressed in the years ahead.

References

1. Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML, Heine-Suñer D, Cigudosa JC, Urioste M, Benitez J, Boix-Chornet M, Sanchez-Aguilera A, Ling C, Carlsson E, Poulsen P, Vaag A, Stephan Z, Spector TD, Wu YZ, Plass C, Esteller M (2005) Epigenetic differences arise during the lifetime of monozygotic twins. Proc Natl Acad Sci USA 102:10604–10609
2. Guil S, Esteller M (2009) DNA methylomes, histone codes and miRNAs: tying it all together. Int J Biochem Cell Biol 41:87–95
3. Reik W, Dean W (2001) DNA methylation and mammalian epigenetics. Electrophoresis 22:2838–2843
4. Morgan HD, Santos F, Green K, Dean W, Reik W (2005) Epigenetic reprogramming in mammals. Hum Mol Genet 14(Suppl 1):R47–R58
5. Bestor TH, Verdine GL (1994) DNA methyltransferases. Curr Opin Cell Biol 6:380–389
6. McClelland M, Ivarie R (1982) Asymmetrical distribution of CpG in an 'average' mammalian gene. Nucleic Acids Res 10:7865–7877
7. Bird AP (1986) CpG-rich islands and the function of DNA methylation. Nature 321:209–213
8. Bestor TH (2000) The DNA methyltransferases of mammals. Hum Mol Genet 9:2395–2402
9. Zhou X, Li Q, Arita A, Sun H, Costa M (2009) Effects of nickel, chromate, and arsenite on histone 3 lysine methylation. Toxicol Appl Pharmacol 236:78–84

10. Zhu B, Zheng Y, Hess D, Angliker H, Schwarz S, Siegmann M, Thiry S, Jost JP (2000) 5-Methylcytosine-DNA glycosylase activity is present in a cloned G/T mismatch DNA glycosylase associated with the chicken embryo DNA methylation complex. Proc Natl Acad Sci USA 97:5135–5139
11. Bennett MT, Rodgers MT, Hebert AS, Ruslander LE, Eisele L, Drohat AC (2006) Specificity of human thymine DNA glycosylase depends on N-glycosidic bond stability. J Am Chem Soc 128:12510–12519
12. Razin A (1998) CpG methylation, chromatin structure and gene silencing—a three way connection. EMBO J 17:4905–4908
13. Razin A, Cedar H (1977) Distribution of 5-methylcytosine in chromatin. Proc Natl Acad Sci USA 74:2725–2728
14. Eckhardt F, Lewin J, Cortese R, Rakyan VK, Attwood J, Burger M, Burton J, Cox TV, Davies R, Down TA, Haefliger C, Horton R, Howe K, Jackson DK, Kunde J, Koenig C, Liddle J, Niblett D, Otto T, Pettett R, Seemann S, Thompson C, West T, Rogers J, Olek A, Berlin K, Beck S (2006) DNA methylation profiling of human chromosomes 6, 20 and 22. Nat Genet 38:1378–1385
15. Finch JT, Lutter LC, Rhodes D, Brown RS, Rushton B, Levitt M, Klug A (1977) Structure of nucleosome core particles of chromatin. Nature 269:29–36
16. Jenuwein T (2001) Re-SET-ting heterochromatin by histone methyltransferases. Trends Cell Biol 11:266–273
17. Wade PA, Pruss D, Wolffe AP (1997) Histone acetylation: chromatin in action. Trends Biochem Sci 22:128–132
18. Karlić R, Chung HR, Lasserre J, Vlahovicek K, Vingron M (2010) Histone modification levels are predictive for gene expression. Proc Natl Acad Sci USA 107:2926–2931
19. Hodawadekar SC, Marmorstein R (2007) Chemistry of acetyl transfer by histone modifying enzymes: structure, mechanism and implications for effector design. Oncogene 26: 5528–5540
20. Hublitz P, Albert M, Peters AH (2010) Mechanisms of transcriptional repression by histone lysine methylation. Int J Dev Biol 53:335–354
21. Shi Y, Whetstine JR (2007) Dynamic regulation of histone lysine methylation by demethylases. Mol Cell 25:1–14
22. Yi T, Fang J, Erdjument-Bromage H, Warren ME, Borchers CH, Tempst P, Zhang Y (2006) Histone demethylation by a family of JmjC domain-containing proteins. Nature 439:811–816
23. Yamane K, Toumazou C, Yi T, Erdjument-Bromage H, Tempst P, Wong J, Zhang Y (2006) JHDM2A, a JmjC-containing H3K9 demethylase, facilitates transcription activation by androgen receptor. Cell 125:483–495
24. Agger K, Christensen J, Cloos PA, Helin K (2008) The emerging functions of histone demethylases. Curr Opin Genet Dev 18:159–168
25. Chuang LSH, Ian HI, Koh TW, Ng HH, Xu G, Li BF (1997) Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for $p21^{WAF1}$. Science 277:1996–2000
26. Iida T, Suetake I, Tajima S, Morioka H, Ohta S, Obuse C, Tsurimoto T (2002) PCNA clamp facilitates action of DNA cytosine methyltransferase 1 on hemimethylated DNA. Genes Cells 7:997–1007
27. Jones PL, Jan Veenstra GC, Wade PA, Vermaak D, Kass SU, Landsberger N, Strouboulis J, Wolffe AP (1998) Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat Genet 19:187–191
28. Schwartz YB, Pirrotta V (2008) Polycomb complexes and epigenetic states. Curr Opin Cell Biol 20:266–273
29. Ringrose L, Paro R (2004) Epigenetic regulation of cellular memory by the polycomb and trithorax group proteins. Annu Rev Genet 38:413–443
30. Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, Jones RS, Zhang Y (2002) Role of histone H3 lysine 27 methylation in polycomb-group silencing. Science 298:1039–1043

31. Cao R, Zhang Y (2004) SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex. Mol Cell 15:57–67
32. Milne TA, Briggs SD, Brock HW, Martin ME, Gibbs D, Allis CD, Hess JL (2002) MLL targets SET domain methyltransferase activity to *Hox* gene promoters. Mol Cell 10: 1107–1117
33. Nakamura T, Mori T, Tada S, Krajewski W, Rozovskaia T, Wassell R, Dubois G, Mazo A, Croce CM, Canaani E (2002) ALL-1 is a histone methyltransferase that assembles a super-complex of proteins involved in transcriptional regulation. Mol Cell 10:1119–1128
34. Okamura K, Lai EC (2008) Endogenous small interfering RNAs in animals. Nat Rev Mol Cell Biol 9:673–678
35. Carthew RW, Sontheimer EJ (2009) Origins and mechanisms of miRNAs and siRNAs. Cell 136:642–655
36. Malone CD, Hannon GJ (2009) Small RNAs as guardians of the genome. Cell 136:656–668
37. Ghildiyal M, Zamore PD (2009) Small silencing RNAs: An expanding universe. Nat Rev Genet 10:94–108
38. Farazi TA, Juranek SA, Tuschl T (2008) The growing catalog of small RNAs and their association with distinct Argonaute/Piwi family members. Development 135:1201–1214
39. Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ (2008) miRBase: tools for microRNA genomics. Nucleic Acids Res 36:D154–D158
40. Berezikov E, Guryev V, van de Belt J, Wienholds E, Plasterk RH, Cuppen E (2005) Phylogenetic shadowing and computational identification of human microRNA genes. Cell 120:21–24
41. Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120:15–20
42. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281–297
43. Hudder A, Novak RF (2008) miRNAs: effectors of environmental influences on gene expression and disease. Toxicol Sci 103:228–240
44. Esquela-Kerscher A, Slack FJ (2006) Oncomirs—microRNAs with a role in cancer. Nat Rev Cancer 6:259–269
45. Lau P, de Strooper B (2010) Dysregulated microRNAs in neurodegenerative disorders. Semin Cell Dev Biol 21:768–773
46. Poy MN, Eliasson L, Krutzfeldt J, Kuwajima S, Ma X, Macdonald PE, Pfeffer S, Tuschl T, Rajewsky N, Rorsman P, Stoffel M (2004) A pancreatic islet-specific microRNA regulates insulin secretion. Nature 432:226–230
47. Sayed D, Hong C, Chen IY, Lypowy J, Abdellatif M (2007) MicroRNAs play an essential role in the development of cardiac hypertrophy. Circ Res 100:416–424
48. Furer V, Greenberg JD, Attur M, Abramson SB, Pillinger MH (2010) The role of microRNA in rheumatoid arthritis and other autoimmune diseases. Clin Immunol 136:1–15
49. He L, Hannon GJ (2004) MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 5:522–531
50. O'Donnell KA, Boeke JD (2007) Mighty Piwis defend the germline against genome intruders. Cell 129:37–44
51. Carmell MA, Girard A, van de Kant HJ, Bourc'his D, Bestor TH, de Rooij DG, Hannon GJ (2007) MIWI2 is essential for spermatogenesis and repression of transposons in the mouse male germline. Dev Cell 12:503–514
52. Aravin AA, Sachidanandam R, Girard A, Fejes-Toth K, Hannon GJ (2007) Developmentally regulated piRNA clusters implicate MILI in transposon control. Science 316:744–747
53. Brennecke J, Aravin AA, Stark A, Dus M, Kellis M, Sachidanandam R, Hannon GJ (2007) Discrete small RNA-generating loci as master regulators of transposon activity in *Drosophila*. Cell 128:1089–1103
54. Aravin AA, Hannon GJ, Brennecke J (2007) The Piwi-piRNA pathway provides an adaptive defense in the transposon arms race. Science 318:761–764

55. Matzke M, Kanno T, Huettel B, Daxinger L, Matzke AJ (2007) Targets of RNA-directed DNA methylation. Curr Opin Plant Biol 10:512–519
56. Bernstein E, Allis CD (2005) RNA meets chromatin. Genes Dev 19:1635–1655
57. Moazed D (2009) Small RNAs in transcriptional gene silencing and genome defence. Nature 457:413–420
58. Zilberman D, Cao X, Jacobsen SE (2003) ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. Science 299:716–719
59. Zilberman D, Cao X, Johansen LK, Xie Z, Carrington JC, Jacobsen SE (2004) Role of *Arabidopsis* ARGONAUTE4 in RNA-directed DNA methylation triggered by inverted repeats. Curr Biol 14:1214–1220
60. Matzke M, Kanno T, Daxinger L, Huettel B, Matzke AJ (2009) RNA-mediated chromatin-based silencing in plants. Curr Opin Cell Biol 21:367–376
61. Verdel A, Jia S, Gerber S, Sugiyama T, Gygi S, Grewal SI, Moazed D (2004) RNAi-mediated targeting of heterochromatin by the RITS complex. Science 303:672–676
62. Motamedi MR, Verdel A, Colmenares SU, Gerber SA, Gygi SP, Moazed D (2004) Two RNAi complexes, RITS and RDRC, physically interact and localize to noncoding centromeric RNAs. Cell 119:789–802
63. Volpe TA, Kidner C, Hall IM, Teng G, Grewal SIS, Martienssen RA (2002) Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science 297: 1833–1837
64. Kim DH, Villeneuve LM, Morris KV, Rossi JJ (2006) Argonaute-1 directs siRNA-mediated transcriptional gene silencing in human cells. Nat Struct Mol Biol 13:793–797
65. Weinberg MS, Villeneuve LM, Ehsani A, Amarzguioui M, Aagaard L, Chen ZX, Riggs AD, Rossi JJ, Morris KV (2006) The antisense strand of small interfering RNAs directs histone methylation and transcriptional gene silencing in human cells. RNA 12:256–262
66. Han J, Kim D, Morris KV (2007) Promoter-associated RNA is required for RNA-directed transcriptional gene silencing in human cells. Proc Natl Acad Sci USA 104:12422–12427
67. Morris KV, Chan SWL, Jacobsen SE, Looney DJ (2004) Small interfering RNA-induced transcriptional gene silencing in human cells. Science 305:1289–1292
68. Kawasaki H, Taira K (2004) Induction of DNA methylation and gene silencing by short interfering RNAs in human cells. Nature 431:211–217
69. Kim DH, Saetrom P, Snøve O Jr, Rossi JJ (2008) MicroRNA-directed transcriptional gene silencing in mammalian cells. Proc Natl Acad Sci USA 105:16230–16235
70. Gonzalez S, Pisano DG, Serrano M (2008) Mechanistic principles of chromatin remodeling guided by siRNAs and miRNAs. Cell Cycle 7:2601–2608
71. Bühler M, Verdel A, Moazed D (2006) Tethering RITS to a nascent transcript initiates RNAi- and heterochromatin-dependent gene silencing. Cell 125:873–886
72. Rybak A, Fuchs H, Smirnova L, Brandt C, Pohl EE, Nitsch R, Wulczyn FG (2008) A feedback loop comprising lin-28 and let-7 controls pre-let-7 maturation during neural stem-cell commitment. Nat Cell Biol 10:987–993
73. Rybak A, Fuchs H, Hadian K, Smirnova L, Wulczyn EA, Michel G, Nitsch R, Krappmann D, Wulczyn FG (2009) The let-7 target gene mouse lin-41 is a stem cell specific E3 ubiquitin ligase for the miRNA pathway protein Ago2. Nat Cell Biol 11:1411–1420
74. Wulczyn FG, Smirnova L, Rybak A, Brandt C, Kwidzinski E, Ninnemann O, Strehle M, Seiler A, Schumacher S, Nitsch R (2007) Post-transcriptional regulation of the let-7 microRNA during neural cell specification. FASEB J 21:415–426
75. Obernosterer G, Leuschner PJ, Alenius M, Martinez J (2006) Post-translational regulation of microRNA expression. RNA 12:1161–1167
76. Weber B, Stresemann C, Brueckner B, Lyko F (2007) Methylation of human microRNA genes in normal and neoplastic cells. Cell Cycle 6:1001–1005
77. Iorio MV, Visone R, Di Leva G, Donati V, Petrocca F, Casalini P, Taccioli C, Volinia S, Liu CG, Alder H, Calin GA, Menard S, Croce CM (2007) MicroRNA signatures in human ovarian cancer. Cancer Res 67:8699–8707

78. Lujambio A, Esteller M (2009) How epigenetics can explain human metastasis: a new role for microRNAs. Cell Cycle 8:377–382
79. Kozaki K, Imoto I, Mogi S, Omura K, Inazawa J (2008) Exploration of tumor-suppressive microRNAs silenced by DNA hypermethylation in oral cancer. Cancer Res 68:2094–2105
80. Saito Y, Liang G, Egger G, Friedman JM, Chuang JC, Coetzee GA, Jones PA (2006) Specific activation of microRNA-127 with downregulation of the proto-oncogene *BCL6* by chromatin-modifying drugs in human cancer cells. Cancer Cell 9:435–443
81. Toyota M, Suzuki H, Sasaki Y, Maruyama R, Imai K, Shinomura Y, Tokino T (2008) Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer. Cancer Res 68:4123–4132
82. Denis H, Ndlovu MN, Fuks F (2011) Regulation of mammalian DNA methyltransferases: a route to new mechanisms. EMBO Rep 12:647–656
83. Fabbri M, Garzon R, Cimmino A, Liu Z, Zanesi N, Callegari E, Liu S, Alder H, Costinean S, Fernandez-Cymering C, Volinia S, Guler G, Morrison CD, Chan KK, Marcucci G, Calin GA, Huebner K, Croce CM (2007) MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. Proc Natl Acad Sci USA 104: 15805–15810
84. Garzon R, Liu S, Fabbri M, Liu Z, Heaphy CE, Callegari E, Schwind S, Pang J, Yu J, Muthusamy N, Havelange V, Volinia S, Blum W, Rush LJ, Perrotti D, Andreeff M, Bloomfield CD, Byrd JC, Chan K, Wu LC, Croce CM, Marcucci G (2009) MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. Blood 113:6411–6418
85. Duursma AM, Kedde M, Schrier M, le Sage C, Agami R (2008) miR-148 targets human DNMT3b protein coding region. RNA 14:872–877
86. Tuddenham L, Wheeler G, Ntounia-Fousara S, Waters J, Hajihosseini MK, Clark I, Dalmay T (2006) The cartilage specific microRNA-140 targets histone deacetylase 4 in mouse cells. FEBS Lett 580:4214–4217
87. Chen JF, Mandel EM, Thomson JM, Wu Q, Callis TE, Hammond SM, Conlon FL, Wang DZ (2006) The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat Genet 38:228–233
88. Winbanks CE, Wang B, Beyer C, Koh P, White L, Kantharidis P, Gregorevic P (2011) TGF-β regulates miR-206 and miR-29 to control myogenic differentiation through regulation of HDAC4. J Biol Chem 286:13805–13814
89. Huang H, Xie C, Sun X, Ritchie RP, Zhang J, Chen YE (2010) miR-10a contributes to retinoid acid-induced smooth muscle cell differentiation. J Biol Chem 285:8383–9389
90. Sinkkonen L, Hugenschmidt T, Berninger P, Gaidatzis D, Mohn F, Artus-Revel CG, Zavolan M, Svoboda P, Filipowicz W (2008) MicroRNAs control *de novo* DNA methylation through regulation of transcriptional repressors in mouse embryonic stem cells. Nat Struct Mol Biol 15:259–267
91. Ashe A, Whitelaw E (2007) Another role for RNA: a messenger across generations. Trends Genet 23:8–10
92. Siemiatycki J, Richardson L, Straif K, Latreille B, Lakhani R, Campbell S, Rousseau MC, Boffetta P (2004) Listing occupational carcinogens. Environ Health Perspect 112: 1447–1459
93. Liang G, Gonzalgo ML, Salem C, Jones PA (2002) Identification of DNA methylation differences during tumorigenesis by methylation-sensitive arbitrarily primed polymerase chain reaction. Methods 27:150–155
94. Gonzalgo ML, Liang G, Spruck CH 3rd, Zingg JM, Rideout WM 3rd, Jones PA (1997) Identification and characterization of differentially methylated regions of genomic DNA by methylation-sensitive arbitrarily primed PCR. Cancer Res 57:594–599
95. Calin GA, Croce CM (2006) MicroRNA signatures in human cancers. Nat Rev Cancer 6: 857–866

96. Zhao CQ, Young MR, Diwan BA, Coogan TP, Waalkes MP (1997) Association of arsenic-induced malignant transformation with DNA hypomethylation and aberrant gene expression. Proc Natl Acad Sci USA 94:10907–10912
97. Cangul H, Broday L, Salnikow K, Sutherland J, Peng W, Zhang Q, Poltaratsky V, Yee H, Zoroddu MA, Costa M (2002) Molecular mechanisms of nickel carcinogenesis. Toxicol Lett 127:69–75
98. Murphy SK, Jirtle RL (2000) Imprinted genes as potential genetic and epigenetic toxicologic targets. Environ Health Perspect 108(Suppl 1):5–11
99. Wareham KA, Lyon MF, Glenister PH, Williams ED (1987) Age related reactivation of an X-linked gene. Nature 327:725–727
100. Cattanach BM (1974) Position effect variegation in the mouse. Genet Res 23:291–306
101. Bennett-Baker PE, Wilkowski J, Burke DT (2003) Age-associated activation of epigenetically repressed genes in the mouse. Genetics 165:2055–2062
102. Park CW, Chung JH (2001) Age-dependent changes of $p57^{Kip2}$ and $p21^{Cip1/Waf1}$ expression in skeletal muscle and lung of mice. Biochim Biophys Acta 1520:163–168
103. Barbot W, Dupressoir A, Lazar V, Heidmann T (2002) Epigenetic regulation of an IAP retrotransposon in the aging mouse: progressive demethylation and de-silencing of the element by its repetitive induction. Nucleic Acids Res 30:2365–2373
104. Wainfan E, Dizik M, Stender M, Christman JK (1989) Rapid appearance of hypomethylated DNA in livers of rats fed cancer-promoting, methyl-deficient diets. Cancer Res 49: 4094–4097
105. Miltenberger RJ, Mynatt RL, Wilkinson JE, Woychik RP (1997) The role of the agouti gene in the yellow obese syndrome. J Nutr 127:1902S–1907S
106. Wolff GL, Kodell RL, Moore SR, Cooney CA (1998) Maternal epigenetics and methyl supplements affect agouti gene expression in A^{vy}/a mice. FASEB J 12:949–957
107. Cooney CA, Dave AA, Wolff GL (2002) Maternal methyl supplements in mice affect epigenetic variation and DNA methylation of offspring. J Nutr 132:2393S–2400S
108. Fraga MF, Agrelo R, Esteller M (2007) Cross-talk between aging and cancer: the epigenetic language. Ann NY Acad Sci 1100:60–74
109. Vertino PM, Issa JP, Pereira-Smith OM, Baylin SB (1994) Stabilization of DNA methyltransferase levels and CpG island hypermethylation precede SV40-induced immortalization of human fibroblasts. Cell Growth Differ 5:1395–1402
110. Choi SW, Mason JB (2000) Folate and carcinogenesis: an integrated scheme. J Nutr 130: 129–132
111. Mehedint MG, Niculescu MD, Craciunescu CN, Zeisel SH (2010) Choline deficiency alters global histone methylation and epigenetic marking at the Re1 site of the calbindin 1 gene. FASEB J 24:184–195
112. Davison JM, Mellott TJ, Kovacheva VP, Blusztajn JK (2009) Gestational choline supply regulates methylation of histone H3, expression of histone methyltransferases G9a (Kmt1c) and Suv39h1 (Kmt1a), and DNA methylation of their genes in rat fetal liver and brain. J Biol Chem 284:1982–1989
113. Tryndyak VP, Ross SA, Beland FA, Pogribny IP (2009) Down-regulation of the microRNAs miR-34a, miR-127, and miR-200b in rat liver during hepatocarcinogenesis induced by a methyl-deficient diet. Mol Carcinog 48:479–487
114. Simone NL, Soule BP, Ly D, Saleh AD, Savage JE, Degraff W, Cook J, Harris CC, Gius D, Mitchell JB (2009) Ionizing radiation-induced oxidative stress alters miRNA expression. PLoS One 4:e6377
115. Cheng Y, Liu X, Zhang S, Lin Y, Yang J, Zhang C (2009) MicroRNA-21 protects against H_2O_2-induced injury on cardiac myocytes via its target gene *PDCD4*. J Mol Cell Cardiol 47:5–14
116. Wagner-Ecker M, Schwager C, Wirkner U, Abdollahi A, Huber PE (2010) MicroRNA expression after ionizing radiation in human endothelial cells. Radiat Oncol 5:25–34
117. Oh JS, Kim JJ, Byun JY, Kim IA (2010) Lin28-let7 modulates radiosensitivity of human cancer cells with activation of K-Ras. Int J Radiat Oncol Biol Phys 76:5–8

118. Chen G, Zhu W, Shi D, Lv L, Zhang C, Liu P, Hu W (2010) MicroRNA-181a sensitizes human malignant glioma U87MG cells to radiation by targeting Bcl-2. Oncol Rep 23: 997–1003
119. Cha HJ, Seong KM, Bae S, Jung JH, Kim CS, Yang KH, Jin YW, An S (2009) Identification of specific microRNAs responding to low and high dose γ-irradiation in the human lymphoblast line IM9. Oncol Rep 22:863–868
120. Rocha S (2007) Gene regulation under low oxygen: holding your breath for transcription. Trends Biochem Sci 32:389–397
121. Kenneth NS, Rocha S (2008) Regulation of gene expression by hypoxia. Biochem J 414: 19–29
122. Wilmink GJ, Roth CL, Ibey BL, Ketchum N, Bernhard J, Cerna CZ, Roach WP (2010) Identification of microRNAs associated with hyperthermia-induced cellular stress response. Cell Stress Chaperones 15:1027–1038
123. Dresios J, Aschrafi A, Owens GC, Vanderklish PW, Edelman GM, Mauro VP (2005) Cold stress-induced protein Rbm3 binds 60S ribosomal subunits, alters microRNA levels, and enhances global protein synthesis. Proc Natl Acad Sci USA 102:1865–1870
124. Marsit CJ, Eddy K, Kelsey KT (2006) MicroRNA responses to cellular stress. Cancer Res 66:10843–10848
125. Vasudevan S, Tong Y, Steitz JA (2007) Switching from repression to activation: microRNAs can up-regulate translation. Science 318:1931–1934
126. Strum JC, Johnson JH, Ward J, Xie H, Feild J, Hester A, Alford A, Waters KM (2009) MicroRNA 132 regulates nutritional stress-induced chemokine production through repression of SirT1. Mol Endocrinol 23:1876–1884
127. Gaedicke S, Zhang X, Schmelzer C, Lou Y, Doering F, Frank J, Rimbach G (2008) Vitamin E dependent microRNA regulation in rat liver. FEBS Lett 582:3542–3546
128. Zhang J, Zhang F, Didelot X, Bruce KD, Cagampang FR, Vatish M, Hanson M, Lehnert H, Ceriello A, Byrne CD (2009) Maternal high fat diet during pregnancy and lactation alters hepatic expression of insulin like growth factor-2 and key microRNAs in the adult offspring. BMC Genomics 10:478–489
129. Stuart TP (1882) Nickel and cobalt: their physiological action on the animal organism. Part I. Toxicology. J Anat Physiol 17:89–123
130. Klein CB, Conway K, Wang XW, Bhamra RK, Lin XH, Cohen MD, Annab L, Barrett JC, Costa M (1991) Senescence of nickel-transformed cells by an X chromosome: possible epigenetic control. Science 251:796–799
131. Wang XW, Lin X, Klein CB, Bhamra RK, Lee YW, Costa M (1992) A conserved region in human and Chinese hamster X chromosomes can induce cellular senescence of nickel-transformed Chinese hamster cell lines. Carcinogenesis 13:555–561
132. Sen P, Costa M (1985) Induction of chromosomal damage in Chinese hamster ovary cells by soluble and particulate nickel compounds: preferential fragmentation of the heterochromatic long arm of the X-chromosome by carcinogenic crystallite NiS particles. Cancer Res 45:2320–2325
133. Conway K, Wang XW, Xu LS, Costa M (1987) Effect of magnesium on nickel-induced genotoxicity and cell transformation. Carcinogenesis 8:1115–1121
134. Sen P, Costa M (1986) Pathway of nickel uptake influences its interaction with heterochromatic DNA. Toxicol Appl Pharmacol 84:278–285
135. Ellen TP, Kluz T, Harder ME, Xiong J, Costa M (2009) Heterochromatinization as a potential mechanism of nickel-induced carcinogenesis. Biochemistry 48:4626–4632
136. Lee YW, Klein CB, Kargacin B, Salnikow K, Kitahara J, Dowjat K, Zhitkovich A, Christie NT, Costa M (1995) Carcinogenic nickel silences gene expression by chromatin condensation and DNA methylation: a new model for epigenetic carcinogens. Mol Cell Biol 15:2547–2557
137. Govindarajan B, Klafter R, Miller MS, Mansur C, Mizesko M, Bai X, LaMontagne K Jr, Arbiser JL (2002) Reactive oxygen-induced carcinogenesis causes hypermethylation of $p16^{Ink4a}$ and activation of MAP kinase. Mol Med 8:1–8

138. Ji W, Yang L, Yu L, Yuan J, Hu D, Zhang W, Yang J, Pang Y, Li W, Lu J, Fu J, Chen J, Lin Z, Chen W, Zhuang Z (2008) Epigenetic silencing of O^6-methylguanine DNA methyltransferase gene in NiS-transformed cells. Carcinogenesis 29:1267–1275
139. Gonzalo V, Lozano JJ, Munoz J, Balaguer F, Pellise M, de Rodriguez MC, Andreu M, Jover R, Llor X, Giraldez MD, Ocana T, Serradesanferm A, Alonso-Espinaco V, Jimeno M, Cuatrecasas M, Sendino O, Castellvi-Bel S, Castells A (2010) Aberrant gene promoter methylation associated with sporadic multiple colorectal cancer. PLoS One 5:e8777
140. Hibi K, Sakata M, Yokomizo K, Kitamura YH, Sakuraba K, Shirahata A, Goto T, Mizukami H, Saito M, Ishibashi K, Kigawa G, Nemoto H, Sanada Y (2009) Methylation of the *MGMT* gene is frequently detected in advanced gastric carcinoma. Anticancer Res 29:5053–5055
141. Zhang J, Zhang J, Li M, Wu Y, Fan Y, Zhou Y, Tan L, Shao Z, Shi H (2011) Methylation of *RAR-β2*, *RASSF1A*, and *CDKN2A* genes induced by nickel subsulfide and nickel-carcinogenesis in rats. Biomed Environ Sci 24:163–171
142. Golebiowski F, Kasprzak KS (2005) Inhibition of core histones acetylation by carcinogenic nickel(II). Mol Cell Biochem 279:133–139
143. Ke Q, Davidson T, Chen H, Kluz T, Costa M (2006) Alterations of histone modifications and transgene silencing by nickel chloride. Carcinogenesis 27:1481–1488
144. Chen H, Ke Q, Kluz T, Yan Y, Costa M (2006) Nickel ions increase histone H3 lysine 9 dimethylation and induce transgene silencing. Mol Cell Biol 26:3728–3737
145. Karaczyn AA, Golebiowski F, Kasprzak KS (2006) Ni(II) affects ubiquitination of core histones H2B and H2A. Exp Cell Res 312:3252–3259
146. Ke Q, Ellen TP, Costa M (2008) Nickel compounds induce histone ubiquitination by inhibiting histone deubiquitinating enzyme activity. Toxicol Appl Pharmacol 228:190–199
147. Chen H, Giri NC, Zhang R, Yamane K, Zhang Y, Maroney M, Costa M (2009) Nickel ions inhibit histone demethylase JMJD1A and DNA repair enzyme ABH2 by replacing the ferrous iron in the catalytic centers. J Biol Chem 285:7374–7383
148. Doll R (1958) Cancer of the lung and nose in nickel workers. Br J Ind Med 15:217–223
149. Doll R, Mathews JD, Morgan LG (1977) Cancers of the lung and nasal sinuses in nickel workers: a reassessment of the period of risk. Br J Ind Med 34:102–105
150. Doll R, Morgan LG, Speizer FE (1970) Cancers of the lung and nasal sinuses in nickel workers. Br J Cancer 24:623–632
151. Ren X, McHale CM, Skibola CF, Smith AH, Smith MT, Zhang L (2011) An emerging role for epigenetic dysregulation in arsenic toxicity and carcinogenesis. Environ Health Perspect 119:11–19
152. Lee-Feldstein A (1986) Cumulative exposure to arsenic and its relationship to respiratory cancer among copper smelter employees. J Occup Med 28:296–302
153. Lee-Feldstein A (1983) Arsenic and respiratory cancer in humans: follow-up of copper smelter employees in Montana. J Natl Cancer Inst 70:601–610
154. Enterline PE, Henderson VL, Marsh GM (1987) Exposure to arsenic and respiratory cancer. A reanalysis. Am J Epidemiol 125:929–938
155. Bertolero F, Pozzi G, Sabbioni E, Saffiotti U (1987) Cellular uptake and metabolic reduction of pentavalent to trivalent arsenic as determinants of cytotoxicity and morphological transformation. Carcinogenesis 8:803–808
156. Lee TC, Oshimura M, Barrett JC (1985) Comparison of arsenic-induced cell transformation, cytotoxicity, mutation and cytogenetic effects in Syrian hamster embryo cells in culture. Carcinogenesis 6:1421–1426
157. DiPaolo JA, Casto BC (1979) Quantitative studies of *in vitro* morphological transformation of Syrian hamster cells by inorganic metal salts. Cancer Res 39:1008–1013
158. Reichard JF, Schnekenburger M, Puga A (2007) Long term low-dose arsenic exposure induces loss of DNA methylation. Biochem Biophys Res Commun 352:188–192
159. Mass MJ, Wang L (1997) Arsenic alters cytosine methylation patterns of the promoter of the tumor suppressor gene *p53* in human lung cells: a model for a mechanism of carcinogenesis. Mutat Res 386:263–277

160. Chanda S, Dasgupta UB, Guhamazumder D, Gupta M, Chaudhuri U, Lahiri S, Das S, Ghosh N, Chatterjee D (2006) DNA hypermethylation of promoter of gene *p53* and *p16* in arsenic-exposed people with and without malignancy. Toxicol Sci 89:431–437
161. Marsit CJ, Karagas MR, Danaee H, Liu M, Andrew A, Schned A, Nelson HH, Kelsey KT (2006) Carcinogen exposure and gene promoter hypermethylation in bladder cancer. Carcinogenesis 27:112–116
162. Huang YC, Hung WC, Chen WT, Yu HS, Chai CY (2009) Sodium arsenite-induced DAPK promoter hypermethylation and autophagy via ERK1/2 phosphorylation in human uroepithelial cells. Chem Biol Interact 181:256–262
163. Majumdar S, Chanda S, Ganguli B, Mazumder DN, Lahiri S, Dasgupta UB (2010) Arsenic exposure induces genomic hypermethylation. Environ Toxicol 25:315–318
164. Ramirez T, Brocher J, Stopper H, Hock R (2008) Sodium arsenite modulates histone acetylation, histone deacetylase activity and HMGN protein dynamics in human cells. Chromosoma 117:147–157
165. Li J, Gorospe M, Barnes J, Liu Y (2003) Tumor promoter arsenite stimulates histone H3 phosphoacetylation of proto-oncogenes *c-fos* and *c-jun* chromatin in human diploid fibroblasts. J Biol Chem 278:13183–13191
166. Li J, Chen P, Sinogeeva N, Gorospe M, Wersto RP, Chrest FJ, Barnes J, Liu Y (2002) Arsenic trioxide promotes histone H3 phosphoacetylation at the chromatin of *CASPASE-10* in acute promyelocytic leukemia cells. J Biol Chem 277:49504–49510
167. Jo WJ, Ren X, Chu F, Aleshin M, Wintz H, Burlingame A, Smith MT, Vulpe CD, Zhang L (2009) Acetylated H4K16 by MYST1 protects UROtsa cells from arsenic toxicity and is decreased following chronic arsenic exposure. Toxicol Appl Pharmacol 241:294–302
168. Zhou X, Sun H, Ellen TP, Chen H, Costa M (2008) Arsenite alters global histone H3 methylation. Carcinogenesis 29:1831–1836
169. Wang Z, Zhao Y, Smith E, Goodall GJ, Drew PA, Brabletz T, Yang C (2011) Reversal and prevention of arsenic-induced human bronchial epithelial cell malignant transformation by microRNA-200b. Toxicol Sci 121:110–122
170. Gregory PA, Bracken CP, Bert AG, Goodall GJ (2008) MicroRNAs as regulators of epithelial-mesenchymal transition. Cell Cycle 7:3112–3118
171. Cui Y, Han Z, Yi H, Song G, Hao C, Xia H, Ma X (2012) MicroRNA-181b and microRNA-9 mediate arsenic-induced angiogenesis via NRP1. J Cell Physiol 227:772–783
172. Cao Y, Yu SL, Wang Y, Guo GY, Ding Q, An RH (2011) MicroRNA-dependent regulation of PTEN after arsenic trioxide treatment in bladder cancer cell line T24. Tumour Biol 32:179–188
173. Dillon CT, Lay PA, Cholewa M, Legge GJ, Bonin AM, Collins TJ, Kostka KL, Shea-McCarthy G (1997) Microprobe X-ray absorption spectroscopic determination of the oxidation state of intracellular chromium following exposure of V79 Chinese hamster lung cells to genotoxic chromium complexes. Chem Res Toxicol 10:533–535
174. Ortega R, Fayard B, Salome M, Deves G, Susini J (2005) Chromium oxidation state imaging in mammalian cells exposed *in vitro* to soluble or particulate chromate compounds. Chem Res Toxicol 18:1512–1519
175. Nickens KP, Patierno SR, Ceryak S (2010) Chromium genotoxicity: a double-edged sword. Chem Biol Interact 188:276–288
176. Klein CB, Su L, Bowser D, Leszczynska J (2002) Chromate-induced epimutations in mammalian cells. Environ Health Perspect 110(Suppl 5):739–743
177. Kondo K, Takahashi Y, Hirose Y, Nagao T, Tsuyuguchi M, Hashimoto M, Ochiai A, Monden Y, Tangoku A (2006) The reduced expression and aberrant methylation of $p16^{INK4a}$ in chromate workers with lung cancer. Lung Cancer 53:295–302
178. Maier A, Dalton TP, Puga A (2000) Disruption of dioxin-inducible phase I and phase II gene expression patterns by cadmium, chromium, and arsenic. Mol Carcinog 28:225–235
179. Wei YD, Tepperman K, Huang MY, Sartor MA, Puga A (2004) Chromium inhibits transcription from polycyclic aromatic hydrocarbon-inducible promoters by blocking the release of histone deacetylase and preventing the binding of p300 to chromatin. J Biol Chem 279:4110–4119

180. Schnekenburger M, Talaska G, Puga A (2007) Chromium cross-links histone deacetylase 1-DNA methyltransferase 1 complexes to chromatin, inhibiting histone-remodeling marks critical for transcriptional activation. Mol Cell Biol 27:7089–7101
181. Takahashi Y, Kondo K, Hirose T, Nakagawa H, Tsuyuguchi M, Hashimoto M, Sano T, Ochiai A, Monden Y (2005) Microsatellite instability and protein expression of the DNA mismatch repair gene, *hMLH1*, of lung cancer in chromate-exposed workers. Mol Carcinog 42:150–158
182. Sun H, Zhou X, Chen H, Li Q, Costa M (2009) Modulation of histone methylation and *MLH1* gene silencing by hexavalent chromium. Toxicol Appl Pharmacol 237:258–266
183. Stanley JS, Griffin JB, Zempleni J (2001) Biotinylation of histones in human cells. Effects of cell proliferation. Eur J Biochem 268:5424–5429
184. Bao B, Pestinger V, Hassan YI, Borgstahl GE, Kolar C, Zempleni J (2011) Holocarboxylase synthetase is a chromatin protein and interacts directly with histone H3 to mediate biotinylation of K9 and K18. J Nutr Biochem 22:470–475
185. Hymes J, Fleischhauer K, Wolf B (1995) Biotinylation of histones by human serum biotinidase: assessment of biotinyl-transferase activity in sera from normal individuals and children with biotinidase deficiency. Biochem Mol Med 56:76–83
186. Xia B, Yang LQ, Huang HY, Pang L, Hu GH, Liu QC, Yuan JH, Liu JJ, Xia YB, Zhuang ZX (2011) Chromium(VI) causes down regulation of biotinidase in human bronchial epithelial cells by modification of histone acetylation. Toxicol Lett 205:140–145
187. Waalkes MP (2003) Cadmium carcinogenesis. Mutat Res 533:107–120
188. Waisberg M, Joseph P, Hale B, Beyersmann D (2003) Molecular and cellular mechanisms of cadmium carcinogenesis. Toxicology 192:95–117
189. Filipič M, Hei TK (2004) Mutagenicity of cadmium in mammalian cells: implication of oxidative DNA damage. Mutat Res 546:81–91
190. Rossman TG, Roy NK, Lin WC (1992) Is cadmium genotoxic? IARC Sci Publ 118:367–375
191. Takiguchi M, Achanzar WE, Qu W, Li G, Waalkes MP (2003) Effects of cadmium on DNA-(cytosine-5) methyltransferase activity and DNA methylation status during cadmium-induced cellular transformation. Exp Cell Res 286:355–365
192. Huang D, Zhang Y, Qi Y, Chen C, Ji W (2008) Global DNA hypomethylation, rather than reactive oxygen species (ROS), a potential facilitator of cadmium-stimulated K562 cell proliferation. Toxicol Lett 179:43–47
193. Jiang G, Xu L, Song S, Zhu C, Wu Q, Zhang L, Wu L (2008) Effects of long-term low-dose cadmium exposure on genomic DNA methylation in human embryo lung fibroblast cells. Toxicology 244:49–55
194. Benbrahim-Tallaa L, Waterland RA, Dill AL, Webber MM, Waalkes MP (2007) Tumor suppressor gene inactivation during cadmium-induced malignant transformation of human prostate cells correlates with overexpression of *de novo* DNA methyltransferase. Environ Health Perspect 115:1454–1459
195. Zhang J, Fu Y, Li J, Wang J, He B, Xu S (2009) Effects of subchronic cadmium poisoning on DNA methylation in hens. Environ Toxicol Pharmacol 27:345–349
196. Zhu H, Li K, Liang J, Zhang J, Wu Q (2011) Changes in the levels of DNA methylation in testis and liver of SD rats neonatally exposed to 5-aza-2′-deoxycytidine and cadmium. J Appl Toxicol 31:484–495
197. Onishchenko N, Tamm C, Vahter M, Hökfelt T, Johnson JA, Johnson DA, Ceccatelli S (2007) Developmental exposure to methylmercury alters learning and induces depression-like behavior in male mice. Toxicol Sci 97:428–437
198. Onishchenko N, Karpova N, Sabri F, Castrén E, Ceccatelli S (2008) Long-lasting depression-like behavior and epigenetic changes of *BDNF* gene expression induced by perinatal exposure to methylmercury. J Neurochem 106:1378–1387
199. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB (2003) Prediction of mammalian microRNA targets. Cell 115:787–798
200. Gorski JA, Zeiler SR, Tamowski S, Jones KR (2003) Brain-derived neurotrophic factor is required for the maintenance of cortical dendrites. J Neurosci 23:6856–6865

201. Desaulniers D, Xiao GH, Lian H, Feng YL, Zhu J, Nakal J, Bowers WJ (2009) Effects of mixtures of polychlorinated biphenyls, methylmercury, and organochlorine pesticides on hepatic DNA methylation in prepubertal female Sprague-Dawley rats. Int J Toxicol 28: 294–307
202. Alexandrov PN, Zhao Y, Pogue AI, Tarr MA, Kruck TP, Percy ME, Cui JG, Lukiw WJ (2005) Synergistic effects of iron and aluminum on stress-related gene expression in primary human neural cells. J Alzheimers Dis 8:117–127
203. Pogue AI, Li YY, Cui JG, Zhao Y, Kruck TP, Percy ME, Tarr MA, Lukiw WJ (2009) Characterization of an NF-κB-regulated, miRNA-146a-mediated down-regulation of complement factor H (CFH) in metal-sulfate-stressed human brain cells. J Inorg Biochem 103: 1591–1595
204. Lukiw WJ, Pogue AI (2007) Induction of specific micro RNA (miRNA) species by ROS-generating metal sulfates in primary human brain cells. J Inorg Biochem 101:1265–1269
205. Sasaki H, Matsui Y (2008) Epigenetic events in mammalian germ-cell development: reprogramming and beyond. Nat Rev Genet 9:129–140
206. Corry GN, Tanasijevic B, Barry ER, Krueger W, Rasmussen TP (2009) Epigenetic regulatory mechanisms during preimplantation development. Birth Defects Res C Embryo Today 87:297–313
207. Calafat AM, Kuklenyik Z, Reidy JA, Caudill SP, Ekong J, Needham LL (2005) Urinary concentrations of bisphenol A and 4-nonylphenol in a human reference population. Environ Health Perspect 113:391–395
208. Ho SM, Tang WY, Belmonte de Frausto J, Prins GS (2006) Developmental exposure to estradiol and bisphenol A increases susceptibility to prostate carcinogenesis and epigenetically regulates phosphodiesterase type 4 variant 4. Cancer Res 66:5624–5632
209. Dolinoy DC, Huang D, Jirtle RL (2007) Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development. Proc Natl Acad Sci USA 104:13056–13061
210. Yaoi T, Itoh K, Nakamura K, Ogi H, Fujiwara Y, Fushiki S (2008) Genome-wide analysis of epigenomic alterations in fetal mouse forebrain after exposure to low doses of bisphenol A. Biochem Biophys Res Commun 376:563–567
211. Bromer JG, Zhou Y, Taylor MB, Doherty L, Taylor HS (2010) Bisphenol-A exposure *in utero* leads to epigenetic alterations in the development programming of uterine estrogen response. FASEB J 24:2273–2280
212. Doherty LF, Bromer JG, Zhou Y, Aldad TS, Taylor HS (2010) *In utero* exposure to diethylstilbestrol (DES) or bisphenol-A (BPA) increases EZH2 expression in the mammary gland: an epigenetic mechanism linking endocrine disruptors to breast cancer. Horm Cancer 1:146–155
213. Kleer CG, Cao Q, Varambally S, Shen R, Ota I, Tomlins SA, Ghosh D, Sewalt RG, Otte AP, Hayes DF, Sabel MS, Livant D, Weiss SJ, Rubin MA, Chinnaiyan AM (2003) EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. Proc Natl Acad Sci USA 100:11606–11611
214. Doshi T, Mehta SS, Dighe V, Balasinor N, Vanage G (2011) Hypermethylation of estrogen receptor promoter region in adult testis of rats exposed neonatally to bisphenol A. Toxicology 289:74–82
215. Avissar-Whiting M, Veiga K, Uhl K, Maccani M, Gagne L, Moen E, Marsit CJ (2010) Bisphenol A exposure leads to specific microRNA alterations in placental cells. Reprod Toxicol 29:401–406
216. Taganov KD, Boldin MP, Chang KJ, Baltimore D (2006) NF-κB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci USA 103:12481–12486
217. Newbold RR, Padilla-Banks E, Jefferson WN (2006) Adverse effects of the model environmental estrogen diethylstilbestrol are transmitted to subsequent generations. Endocrinology 147(Suppl 6):S11–S17

218. Lu LJ, Liehr JG, Sirbasku DA, Randerath E, Randerath K (1988) Hypomethylation of DNA in estrogen-induced and -dependent hamster kidney tumors. Carcinogenesis 9:925–929
219. Li S, Washburn KA, Moore R, Uno T, Teng C, Newbold RR, McLachlan JA, Negishi M (1997) Developmental exposure to diethylstilbestrol elicits demethylation of estrogen-responsive lactoferrin gene in mouse uterus. Cancer Res 57:4356–4359
220. Tang WY, Newbold R, Mardilovich K, Jefferson W, Cheng RY, Medvedovic M, Ho SM (2008) Persistent hypomethylation in the promoter of nucleosomal binding protein 1 (Nsbp1) correlates with overexpression of Nsbp1 in mouse uteri neonatally exposed to diethylstilbestrol or genistein. Endocrinology 149:5922–5931
221. Sato K, Fukata H, Kogo Y, Ohgane J, Shiota K, Mori C (2009) Neonatal exposure to diethylstilbestrol alters expression of DNA methyltransferases and methylation of genomic DNA in the mouse uterus. Endocr J 56:131–139
222. Sato K, Fukata H, Kogo Y, Ohgane J, Shiota K, Mori C (2006) Neonatal exposure to diethylstilbestrol alters the expression of DNA methyltransferases and methylation of genomic DNA in the epididymis of mice. Endocr J 53:331–337
223. Bredfeldt TG, Greathouse KL, Safe SH, Hung MC, Bedford MT, Walker CL (2010) Xenoestrogen-induced regulation of EZH2 and histone methylation via estrogen receptor signaling to PI3K/AKT. Mol Endocrinol 24:993–1006
224. Cha TL, Zhou BP, Xia W, Wu Y, Yang CC, Chen CT, Ping B, Otte AP, Hung MC (2005) Akt-mediated phosphorylation of EZH2 suppresses methylation of lysine 27 in histone H3. Science 310:306–310
225. Hsu PY, Deatherage DE, Rodriguez BA, Liyanarachchi S, Weng YI, Zuo T, Liu J, Cheng AS, Huang TH (2009) Xenoestrogen-induced epigenetic repression of microRNA-9-3 in breast epithelial cells. Cancer Res 69:5936–5945
226. Kelce WR, Monosson E, Gamcsik MP, Laws SC, Gray LE Jr (1994) Environmental hormone disruptors: evidence that vinclozolin developmental toxicity is mediated by antiandrogenic metabolites. Toxicol Appl Pharmacol 126:276–285
227. Anway MD, Cupp AS, Uzumcu M, Skinner MK (2005) Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 308:1466–1469
228. Chang HS, Anway MD, Rekow SS, Skinner MK (2006) Transgenerational epigenetic imprinting of the male germline by endocrine disruptor exposure during gonadal sex determination. Endocrinology 147:5524–5541
229. Schneider S, Kaufmann W, Buesen R, van Ravenzwaay B (2008) Vinclozolin—the lack of a transgenerational effect after oral maternal exposure during organogenesis. Reprod Toxicol 25:352–360
230. Crews D, Gore AC, Hsu TS, Dangleben NL, Spinetta M, Schallert T, Anway MD, Skinner MK (2007) Transgenerational epigenetic imprints on mate preference. Proc Natl Acad Sci USA 104:5942–5946
231. Nilsson EE, Anway MD, Stanfield J, Skinner MK (2008) Transgenerational epigenetic effects of the endocrine disruptor vinclozolin on pregnancies and female adult onset disease. Reproduction 135:713–721
232. Anway MD, Leathers C, Skinner MK (2006) Endocrine disruptor vinclozolin induced epigenetic transgenerational adult-onset disease. Endocrinology 147:5515–5523
233. Guerrero-Bosagna C, Settles M, Lucker B, Skinner MK (2010) Epigenetic transgenerational actions of vinclozolin on promoter regions of the sperm epigenome. PLoS One 5:e13100
234. Skinner MK, Manikkam M, Guerrero-Bosagna C (2011) Epigenetic transgenerational actions of endocrine disruptors. Reprod Toxicol 31:337–343
235. Zama AM, Uzumcu M (2009) Fetal and neonatal exposure to the endocrine disruptor methoxychlor causes epigenetic alterations in adult ovarian genes. Endocrinology 150: 4681–4691
236. Cupp AS, Uzumcu M, Suzuki H, Dirks K, Phillips B, Skinner MK (2003) Effect of transient embryonic *in vivo* exposure to the endocrine disruptor methoxychlor on embryonic and postnatal testis development. J Androl 24:736–745

237. Uzumcu M, Suzuki H, Skinner MK (2008) Effect of the anti-androgenic endocrine disruptor vinclozolin on embryonic testis cord formation and postnatal testis development and function. Reprod Toxicol 18:765–774
238. Stouder C, Paoloni-Giacobino A (2010) Transgenerational effects of the endocrine disruptor vinclozolin on the methylation pattern of imprinted genes in the mouse sperm. Reproduction 139:373–379
239. Peters A (2005) Particulate matter and heart disease: evidence from epidemiological studies. Toxicol Appl Pharmacol 207(Suppl 2):477–482
240. Franchini M, Mannucci PM (2009) Particulate air pollution and cardiovascular risk: short-term and long-term effects. Semin Thromb Hemost 35:665–670
241. Izzotti A, Parodi S, Quaglia A, Fare C, Vercelli M (2000) The relationship between urban airborne pollution and short-term mortality: quantitative and qualitative aspects. Eur J Epidemiol 16:1027–1034
242. Vineis P, Husgafvel-Pursiainen K (2005) Air pollution and cancer: biomarker studies in human populations. Carcinogenesis 26:1846–1855
243. Li N, Xia T, Nel AE (2008) The role of oxidative stress in ambient particulate matter-induced lung diseases and its implications in the toxicity of engineered nanoparticles. Free Radic Biol Med 44:1689–1699
244. Hou M, Morishita Y, Iljima T, Inadome Y, Mase K, Dai Y, Noguchi M (1999) DNA methylation and expression of $p16^{INK4A}$ gene in pulmonary adenocarcinomas and anthracosis in background lung. Int J Cancer 84:609–613
245. Belinsky SA, Snow SS, Nikula KJ, Finch GL, Tellez CS, Palmisano WA (2002) Aberrant CpG island methylation of the $p16^{INK4a}$ and estrogen receptor genes in rat lung tumors induced by particulate carcinogens. Carcinogenesis 23:335–339
246. Hou L, Zhang X, Tarantini L, Nordio F, Bonzini M, Angelici L, Marinelli B, Rizzo G, Cantone L, Apostoli P, Bertazzi PA, Baccarelli A (2011) Ambient PM exposure and DNA methylation in tumor suppressor genes: a cross-sectional study. Part Fibre Toxicol 8:25–34
247. Baccarelli A, Wright RO, Bollati V, Tarantini L, Litonjua AA, Suh HH, Zanobetti A, Sparrow D, Vokonas PS, Schwartz J (2009) Rapid DNA methylation changes after exposure to traffic particles. Am J Respir Crit Care Med 179:572–578
248. Tarantini L, Bonzini M, Apostoli P, Pegoraro V, Bollati V, Marinelli B, Cantone L, Rizzo G, Hou L, Schwartz J, Bertazzi PA, Baccarelli A (2009) Effects of particulate matter on genomic DNA methylation content and iNOS promoter methylation. Environ Health Perspect 117:217–222
249. Borm PJ, Kelly F, Kunzli N, Schins RP, Donaldson K (2007) Oxidant generation by particulate matter: from biologically effective dose to a promising, novel metric. Occup Environ Med 64:73–74
250. Valinluck V, Tsai HH, Rogstad DK, Burdzy A, Bird A, Sowers LC (2004) Oxidative damage to methyl-CpG sequences inhibits the binding of the methyl-CpG binding domain (MBD) of methyl-CpG binding protein 2 (MeCP2). Nucleic Acids Res 32:4100–4108
251. Zhen J, Lu H, Wang XQ, Vaziri ND, Zhou XJ (2008) Upregulation of endothelial and inducible nitric oxide synthase expression by reactive oxygen species. Am J Hypertens 21: 28–34
252. Cantone L, Nordio F, Hou L, Apostoli P, Bonzini M, Tarantini L, Angelici L, Bollati V, Zanobetti A, Schwartz J, Bertazzi PA, Baccarelli A (2011) Inhalable metal-rich air particles and histone H3K4 dimethylation and H3K9 acetylation in a cross-sectional study of steel workers. Environ Health Perspect 119:964–969
253. Bollati V, Marinelli B, Apostoli P, Bonzini M, Nordio F, Hoxha M, Pegoraro V, Motta V, Tarantini L, Cantone L, Schwartz J, Bertazzi PA, Baccarelli A (2010) Exposure to metal-rich particulate matter modifies the expression of candidate microRNAs in peripheral blood leukocytes. Environ Health Perspect 118:763–768
254. Babar IA, Slack FJ, Weidhaas JB (2008) miRNA modulation of the cellular stress response. Future Oncol 4:289–298

255. Xiao C, Rajewsky K (2009) MicroRNA control in the immune system: basic principles. Cell 136:26–36
256. Jardim MJ, Fry RC, Jaspers I, Dailey L, Diaz-Sanchez D (2009) Disruption of microRNA expression in human airway cells by diesel exhaust particles is linked to tumorigenesis-associated pathways. Environ Health Perspect 117:1745–1751
257. Farraj AK, Hazari MS, Haykal-Coates N, Lamb C, Winsett DW, Ge Y, Ledbetter AD, Carll AP, Bruno M, Ghio A, Costa DL (2011) ST depression, arrhythmia, vagal dominance, and reduced cardiac micro-RNA in particulate-exposed rats. Am J Respir Cell Mol Biol 44:185–196
258. Latronico MV, Condorelli G (2009) MicroRNAs and cardiac pathology. Nat Rev Cardiol 6:419–429
259. Condorelli G, Latronico MV, Dorn GW 2nd (2010) microRNAs in heart disease: putative novel therapeutic targets. Eur Heart J 31:649–658
260. Osada H, Takahashi T (2002) Genetic alterations of multiple tumor suppressors and oncogenes in the carcinogenesis and progression of lung cancer. Oncogene 21:7421–7434
261. Schwartz AG, Prysak GM, Bock CH, Cote ML (2007) The molecular epidemiology of lung cancer. Carcinogenesis 28:507–518
262. Belinsky SA, Nikula KJ, Palmisano WA, Michels R, Saccomanno G, Gabrielson E, Baylin SB, Herman JG (1998) Aberrant methylation of $p16^{INK4a}$ is an early event in lung cancer and a potential biomarker for early diagnosis. Proc Natl Acad Sci USA 95:11891–11896
263. Palmisano WA, Divine KK, Saccomanno G, Gilliland FD, Baylin SB, Herman JG, Belinsky SA (2000) Predicting lung cancer by detecting aberrant promoter methylation in sputum. Cancer Res 60:5954–5958
264. Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG (1999) Inactivation of the DNA repair gene *O^6-methylguanine-DNA methyltransferase* by promoter hypermethylation is a common event in primary human neoplasia. Cancer Res 59:793–797
265. Belinsky SA, Palmisano WA, Gilliland FD, Crooks LA, Divine KK, Winters SA, Grimes MJ, Harms HJ, Tellez CS, Smith TM, Moots PP, Lechner JF, Stidley CA, Crowell RE (2002) Aberrant promoter methylation in bronchial epithelium and sputum from current and former smokers. Cancer Res 62:2370–2377
266. Liu F, Killian JK, Yang M, Walker RL, Hong JA, Zhang M, Davis S, Zhang Y, Hussain M, Xi S, Rao M, Meltzer PA, Schrump DS (2010) Epigenomic alterations and gene expression profiles in respiratory epithelia exposed to cigarette smoke condensate. Oncogene 29:3650–3664
267. Pulling LC, Vuillemenot BR, Hutt JA, Devereux TR, Belinsky SA (2004) Aberrant promoter hypermethylation of the death-associated protein kinase gene is early and frequent in murine lung tumors induced by cigarette smoke and tobacco carcinogens. Cancer Res 64:3844–3848
268. Izzotti A, Calin GA, Steele VE, Cartiglia C, Longobardi M, Croce CM, De Flora S (2010) Chemoprevention of cigarette smoke-induced alterations of microRNA expression in rat lungs. Cancer Prev Res 3:62–72
269. Izzotti A, Calin GA, Steele VE, Croce CM, De Flora S (2009) Relationships of microRNA expression in mouse lung with age and exposure to cigarette smoke and light. FASEB J 23:3243–3250
270. Izzotti A, Calin GA, Arrigo P, Steele VE, Croce CM, De Flora S (2009) Downregulation of microRNA expression in the lungs of rats exposed to cigarette smoke. FASEB J 23:806–812
271. Izzotti A, Larghero P, Cartiglia C, Longobardi M, Pfeffer U, Steele VE, De Flora S (2010) Modulation of microRNA expression by budesonide, phenethyl isothiocyanate and cigarette smoke in mouse liver and lung. Carcinogenesis 31:894–901
272. Izzotti A, Bagnasco M, Cartiglia C, Longobardi M, Balansky RM, Merello A, Lubet RA, De Flora S (2005) Chemoprevention of genome, transcriptome, and proteome alterations induced by cigarette smoke in rat lung. Eur J Cancer 41:1864–1874
273. Schembri F, Sridhar S, Perdomo C, Gustafson AM, Zhang X, Ergun A, Lu J, Liu G, Zhang X, Bowers J, Vaziri C, Ott K, Sensinger K, Collins JJ, Brody JS, Getts R, Lenburg ME, Spira A (2009) MicroRNAs as modulators of smoking-induced gene expression changes in human airway epithelium. Proc Natl Acad Sci USA 106:2319–2324

274. Hecht SS (2006) Cigarette smoking: cancer risks, carcinogens, and mechanisms. Langenbecks Arch Surg 391:603–613
275. Kalscheuer S, Zhang X, Zeng Y, Upadhyaya P (2008) Differential expression of microRNAs in early-stage neoplastic transformation in the lungs of F344 rats chronically treated with the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Carcinogenesis 29:2394–2399
276. Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, Stephens RM, Okamoto A, Yokota J, Tanaka T, Calin GA, Liu CG, Croce CM, Harris CC (2006) Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 9:189–198
277. Glaheta RA, Cinatl J Jr (2002) Anti-tumor mechanisms of valproate: a novel role for an old drug. Med Res Rev 22:492–511
278. Phiel CJ, Zhang F, Huang EY, Guenther MG, Lazar MA, Klein PS (2001) Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. J Biol Chem 276:36734–36741
279. Ornoy A (2009) Valproic acid in pregnancy: how much are we endangering the embryo and fetus? Reprod Toxicol 28:1–10
280. Gurvich N, Berman MG, Wittner BS, Gentleman RC, Klein PS, Green JB (2005) Association of valproate-induced teratogenesis with histone deacetylase inhibition *in vivo*. FASEB J 19:1166–1168
281. Gao FB (2008) Posttranscriptional control of neuronal development by microRNA networks. Trends Neurosci 31:20–26
282. Zhou R, Yuan P, Wang Y, Hunsberger JG, Elkahloun A, Wei Y, Damschroder-Williams P, Du J, Chen G, Manji HK (2009) Evidence for selective microRNAs and their effectors as common long-term targets for the actions of mood stabilizers. Neuropsychopharmacology 34:1395–1405
283. Chen H, Wang N, Burmeister M, McInnis MG (2009) MicroRNA expression changes in lymphoblastoid cell lines in response to lithium treatment. Int J Neuropsychopharmacol 12: 975–981
284. Fukushima T, Hamada Y, Yamada H, Horii I (2007) Changes of micro-RNA expression in rat liver treated by acetaminophen or carbon tetrachloride—regulating role of micro-RNA for RNA expression. J Toxicol Sci 32:401–409
285. Amar PJ, Schiff ER (2007) Acetaminophen safety and hepatotoxicity—where do we go from here? Exp Opin Drug Saf 6:341–355
286. Rood AS, McGavran PD, Aanenson JW, Till JE (2001) Stochastic estimates of exposure and cancer risk from carbon tetrachloride released to the air from the rocky flats plant. Risk Anal 21:675–695
287. Recknagel RO (1967) Carbon tetrachloride hepatotoxicity. Pharmacol Rev 19:145–208
288. Wang K, Zhang S, Marzolf B, Troisch P, Brightman A, Hu Z, Hood LE, Galas DJ (2009) Circulating microRNAs, potential biomarkers for drug-induced liver injury. Proc Natl Acad Sci USA 106:4402–4407
289. Phillips DH (2001) Understanding the genotoxicity of tamoxifen? Carcinogenesis 22: 839–849
290. Tryndyak VP, Muskhelishvili L, Kovalchuk O, Rodriguez-Juarez R, Montgomery B, Churchwell MI, Ross SA, Beland FA, Pogribny IP (2006) Effect of long-term tamoxifen exposure on genotoxic and epigenetic changes in rat liver: implications for tamoxifen-induced hepatocarcinogenesis. Carcinogenesis 27:1713–1720
291. Pogribny IP, Tryndyak VP, Boyko A, Rodriguez-Juarez R, Beland FA, Kovalchuk O (2007) Induction of microRNAome deregulation in rat liver by long-term tamoxifen exposure. Mutat Res 619:30–37
292. Gonzalo S, Garcia-Cao M, Fraga MF, Schotta G, Peters AH, Cotter SE, Eguia R, Dean DC, Esteller M, Jenuwein T, Blasco MA (2005) Role of the RB1 family in stabilizing histone methylation at constitutive heterochromatin. Nat Cell Biol 7:420–428
293. Pogribny IP, Muskhelishvili L, Tryndyak VP, Beland FA (2011) The role of epigenetic events in genotoxic hepatocarcinogenesis induced by 2-acetylaminofluorene. Mutat Res 722:106–113

294. Bagnyukova TV, Tryndyak VP, Montgomery B, Churchwell MI, Karpf AR, James SR, Muskhelishvili L, Beland FA, Pogribny IP (2008) Genetic and epigenetic changes in rat preneoplastic liver tissue induced by 2-acetylaminofluorene. Carcinogenesis 29:638–646
295. Gonzalez FJ, Peters JM, Cattley RC (1998) Mechanism of action of the nongenotoxic peroxisome proliferators: role of the peroxisome proliferator-activator receptor α. J Natl Cancer Inst 90:1702–1709
296. Shah YM, Morimura K, Tang Q, Tanabe T, Takagi M, Gonzalez FJ (2007) Peroxisome proliferator-activated receptor α regulates a microRNA-mediated signaling cascade responsible for hepatocellular proliferation. Mol Cell Biol 27:4238–4247
297. Hammond SM (2006) MicroRNAs as oncogenes. Curr Opin Genet Dev 16:4–9
298. Rossi L, Bonmassar E, Faraoni I (2007) Modification of miR gene expression pattern in human colon cancer cells following exposure to 5-fluorouracil *in vitro*. Pharmacol Res 56:248–253
299. Zhang B, Pan X (2009) RDX induces aberrant expression of microRNAs in mouse brain and liver. Environ Health Perspect 117:231–240
300. Mellios N, Huang HS, Grigorenko A, Rogaev E, Akbarian S (2008) A set of differentially expressed miRNAs, including miR-30a-5p, act as post-transcriptional inhibitors of BDNF in prefrontal cortex. Hum Mol Genet 17:3030–3042
301. Viberg H, Mundy W, Eriksson P (2008) Neonatal exposure to decabrominated diphenyl ether (PBDE 209) results in changes in BDNF, CaMKII and GAP-43 biochemical substrates of neuronal survival, growth, and synaptogenesis. Neurotoxicology 29:152–159
302. Onishchenko N, Karpova N, Sabri F, Castren E, Ceccatelli S (2008) Long-lasting depression-like behavior and epigenetic changes of *BDNF* gene expression induced by perinatal exposure to methylmercury. J Neurochem 106:1378–1387
303. Miranda RC, Pietrzykowski AZ, Tang Y, Sathyan P, Mayfield D, Keshavarzian A, Sampson W, Hereld D (2010) Micro-RNAs: master regulators of ethanol abuse and toxicity? Alcohol Clin Exp Res 34:575–587
304. Pietrzykowski AZ, Friesen RM, Martin GE, Puig SI, Nowak CL, Wynne PM, Siegelmann HT, Treistman SN (2008) Posttranslational regulation of BK channel splice variant stability by miR-9 underlies neuroadaptation to alcohol. Neuron 59:274–287
305. Tang Y, Banan A, Forsyth CB, Fields JZ, Lau CK, Zhang LJ, Keshavarzian A (2008) Effect of alcohol on miR-212 expression in intestinal epithelial cells and its potential role in alcoholic liver disease. Alcohol Clin Exp Res 32:355–364
306. Sathyan P, Golden HB, Miranda RC (2007) Competing interactions between micro-RNAs determine neural progenitor survival and proliferation after ethanol exposure: evidence from an *ex vivo* model of the fetal cerebral cortical neuroepithelium. J Neurosci 27:8546–8557
307. Santillano DR, Kumar LS, Prock TL, Camarillo C, Tingling JD, Miranda RC (2005) Ethanol induces cell-cycle activity and reduces stem cell diversity to alter both regenerative capacity and differentiation potential of cerebral cortical neuroepithelial precursors. BMC Neurosci 6:59–75
308. Wang LL, Zhang Z, Li Q, Yang R, Pei X, Xu Y, Wang J, Zhou SF, Li Y (2009) Ethanol exposure induces differential microRNA and target gene expression and teratogenic effects which can be suppressed by folic acid supplementation. Hum Reprod 24:562–579
309. Van den Heuvel JP, Lucier G (1993) Environmental toxicology of polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans. Environ Health Perspect 100:189–200
310. Moffat ID, Boutros PC, Celius T, Lindén J, Pohjanvirta R, Okey AB (2007) MicroRNAs in adult rodent liver are refractory to dioxin treatment. Toxicol Sci 99:470–487
311. Elyakim E, Sitbon E, Faerman A, Tabak S, Montia E, Belanis L, Dov A, Marcusson EG, Bennett CF, Chajut A, Cohen D, Yerushalmi N (2010) has-miR-191 is a candidate oncogene target for hepatocellular carcinoma therapy. Cancer Res 70:8077–8087
312. Yauk CL, Jackson K, Malowany M, Williams A (2010) Lack of change in microRNA expression in adult mouse liver following treatment with benzo[*a*]pyrene despite robust mRNA transcriptional response. Mutat Res 722:131–139

313. Shen YL, Jiang YG, Greenlee AR, Zhou LL, Liu LH (2009) MicroRNA expression profiles and miR-10a target in *anti*-benzo[*a*]pyrene-7,8-diol-9,10-epoxide-transformed human 16HBE cells. Biomed Environ Sci 22:14–21
314. Debernardi S, Skoulakis S, Molloy G, Chaplin T, Dixon-McIver A, Young BD (2007) MicroRNA miR-181a correlates with morphological sub-class of acute myeloid leukaemia and the expression of its target genes in global genome-wide analysis. Leukemia 21:912–916

Hormesis: Improving Predictions in the Low-Dose Zone

E.J. Calabrese

Abstract This chapter explores the historical foundations of hormesis, including the underlying reasons for its marginalization during most of the twentieth century and factors that are contributing to its resurgence and acceptance within the toxicological and pharmacological communities. Special consideration is given to the quantitative features of the hormetic dose response, as well as its capacity for generalization. Based on subsequent comparisons with other leading dose–response models, the hormesis dose response consistently provides more accurate predictions in the below threshold zone. It is expected that the hormetic dose response will become progressively more useful to the fields of toxicology, pharmacology, risk assessment, and the life sciences in general, especially where low-dose effects are of interest.

Keywords Dose–response relationship · Threshold model · Biphasic dose response · Homeopathy · Inverted U-shape · Adaptive response · Nonmonotonic dose response

Introduction

The discipline of toxicology and its offspring, risk assessment, have as their central pillar, the dose–response relationship. Nearly every fundamental initiative in these fields is centered on this concept. Beliefs about the dose response affect how experiments are designed, what animal models may be selected for study, the

E.J. Calabrese (✉)
Department of Public Health, University of Massachusetts, Amherst, MA, USA
Department of Toxicology, Environmental Health Sciences, University of Massachusetts, Morrill I, N344, Amherst, MA 01003, USA
e-mail: edwardc@schoolph.umass.edu

A. Luch (ed.), *Molecular, Clinical and Environmental Toxicology*,
Experientia Supplementum 101, DOI 10.1007/978-3-7643-8340-4_19,

types of endpoints or diseases assessed, assumptions concerning mechanisms that may account for adverse effects, and how to predict responses, that is, to extrapolate to low-dose exposures to which humans may be exposed. These activities have led to the establishment of large numbers of environmental health standards by the US Environmental Protection Agency (US-EPA) for contaminants in air, water, and soil. The same dose–response methods have also been used by the Occupational Safety and Health Administration (OSHA) to establish industrial health exposure standards and by the Food and Drug Administration (FDA) to protect the public from adverse effects from thousands of drugs and food additives used by society. Likewise, the private sector pharmaceutical industry has used the dose–response relationship as its own central pillar in the design and conduct of essentially all preclinical investigations and clinical trials of vast arrays of approved drugs and those in the evaluation pipeline.

Dose Response: Historical Foundations

These agencies, organizations, and, indeed, the entire biomedical community have built their approaches for assessing the effects of chemicals and pharmaceuticals on the long-standing belief that most, if not all agents, follow a threshold dose–response relationship. This means that at doses above the threshold, biological responses (whether beneficial or harmful) occur, whereas below the threshold the dose is considered too dilute for biological effects to be induced. This belief in the threshold model started nearly a century ago [1–4] became "institutionalized" and has been long taken for granted that it correctly predicts how agents affect all types of biological systems, with no meaningful exceptions [5, 6].

Hormesis: Its Name and Origin

While the above discussion describes past and present dose–response assumptions of the scientific and regulatory communities concerned with the health effects of chemicals and drugs, the core belief in the threshold model has come under severe challenge over the past decade based on reams of data showing that reproducible biological effects often occur at doses below toxic and/or pharmacological thresholds, changing the dose–response relationship from a "threshold" to one with biphasic properties, that is, a low-dose stimulation and a high-dose inhibition [7]. This biphasic dose response is called hormesis (from the Greek meaning to excite), having been so named in 1943 by Chester Southam and John Ehrlich [8], then forestry researchers at the University of Idaho, who observed that extracts from the red cedar tree could inhibit fungal growth at high concentrations while stimulating it at lower concentrations [9, 10].

Historical Blunders

The idea that the fundamental nature of the dose response may be biphasic, that is, hormetic in nature, has a long history. In fact, one can trace its initial formulations back to the late 1880s based on studies in northern Germany by Hugo Schulz [11, 12], a physician and academic pharmacologist, who reported such biphasic dose responses when assessing the effects of various disinfectants on yeast metabolism. However, a problem arose when Schulz, who had a long personal and professional interest in homeopathy due in large part to family friendships [13], thought that his findings provided the underlying scientific foundation for this controversial medical practice. He quickly linked up with leading homeopaths, who were seeking academic credibility, becoming an intellectual leader within this group over the next four decades. Schulz argued that below toxic doses of homeopathic medications induced adaptive responses that enhanced the capacity of patients to resist various diseases and that most agents would be expected to display biphasic dose–response relationships [14].

Since homeopathy was engaged in a prolonged, intense, and very acrimonious competition with what is now called "traditional medicine," Schulz and his dose–response theories became the object of scientific ridicule and became marginalized by leaders in the European medical community, especially well-known, accomplished, and influential pharmacologists such as Alfred J. Clark, chair of pharmacology at Edinburgh. These efforts by Clark and others attempted to link Schulz with the more extremist elements, that is, the high dilutionist wing of the homeopathy field, making him and his biphasic dose–response theory collateral damage in the homeopathy-traditional medicine culture "war" [5]. These efforts to discredit Schulz and therefore his scientific ideas were as successful as they were disingenuous. Lacking in the attacks on Schulz was any acknowledgment that the high dilutionist wing of homeopathy was nonrepresentative of this medical body, since it represented only a very small minority within that medical practice [5, 15]; however, it was easy to attack the extreme positions of this Hahnemann-inspired small minority and then color the entire body of practitioners, including Schulz, with the same brush. Secondly, numerous examples of dose responses similar to those reported by Schulz by other credible scientists with no linkage to homeopathy were never cited in the highly influential writings of Clark [4] (see earlier literature as reviewed in [16–22]).

The guilt by association label that the hormesis concept acquired was unfortunate for the fields of toxicology, pharmacology, and, indeed, all disciplines concerned with dose–response relationships. Hormetic dose–response relationships should not have been a pawn in an economic competition between two medical systems. Hormesis, a dose–response phenomenon that displays a low-dose stimulation and a high-dose inhibition (Fig. 1), with specific quantitative features (as will be discussed in greater detail below) is not the "special" dose response of any medical system, but a scientific concept with important biological implications. Whether such biphasic dose responses could be objectively established and shown to be reproducible was the key issue, a point missed in the battle of medical titans of the early decades of the twentieth century. Nonetheless, the historical foundations that determined which dose response would dominate the twentieth century were determined less by science than by power and politics.

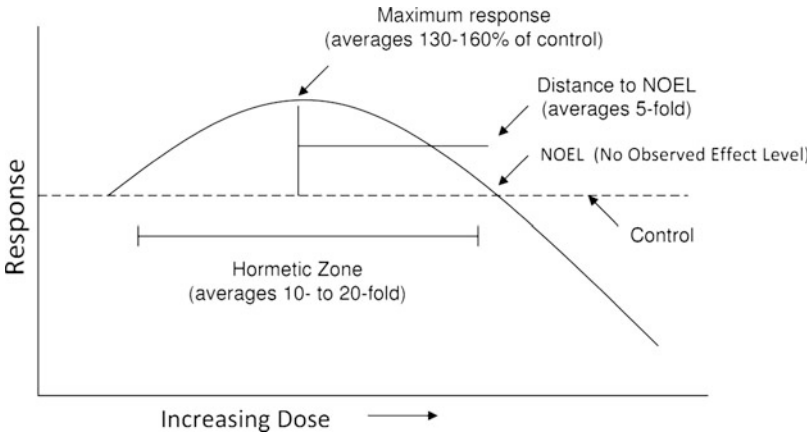


Fig. 1 Dose–response curve depicting the quantitative features of hormesis

Having for all practical intents "killed" acceptance and credibility of the biphasic (later to be named hormesis) dose–response model of Schulz, Clark and the broader pharmacology community made the case for an alternative dose–response model to guide the toxicological and biomedical sciences, and this was in large measure how the threshold model was born and established. It soon became institutionalized in the 1930s during a period of concept consolidation by the scientific and regulatory communities [4]. The entire public health and medical frameworks became based on this foundation, and it remains so even today. The only exception has been in the area of radiation and chemical carcinogenesis in which the threshold model was forced to give way to linearity at low-dose modeling [23, 24] due to society's fear of cancer even though the scientific foundations of the low-dose linear modeling remain impossible to validate low risk estimates (i.e., $1 < 1{,}000$) [9, 10].

Resurgence of Hormesis

By the early 1980s, numerous scientific advances and regulatory conditions had created a framework that would lead to a reexamination of the threshold model and to the current resurgence of interest in hormesis. First, the implementation of linearity at low-dose modeling for cancer risk assessment by US-EPA in the early 1980s based on the recommendation of the National Academy of Sciences (NAS) Safe Drinking Water Committee in 1977 [24] brought enormous new costs to industry since the acceptable exposure standards required cleanup activities to achieve extremely low concentrations. Many in the industrial sector felt such extraordinary remedial activities lacked biomedical justification, and therefore, there was a need to challenge the US-EPA low-dose linearity approach for regulating carcinogen exposures; their initial strategy proposed the replacement of linear at low-dose modeling with the threshold dose–response model since it was likely that all agents, including carcinogens, acted

via thresholds. However, statistical assessments, based on the limited data of individual animal bioassay experiments, could never adequately distinguish the linear from the threshold model. In such cases, the US-EPA would always favor (i.e., default to) the more conservative model, which would be the linear approach.

Realizing that they could never "win" using the threshold model challenge approach, tactics were changed, thinking that the hormesis model might be successful since it could be more readily differentiated from the linear at low-dose model, if only there were data to support it. Thus, in an ironic twist of fate, the extremely conservative approach of the US-EPA for assessing risks from exposures to low doses of carcinogens led to a reexamination of the hormesis dose–response model, its occurrence, frequency, reproducibility, underlying mechanisms, and applications to toxicology and risk assessment. In fact, the first such conference on hormesis was held during August 1985 in Oakland, CA, with a focus on radiation. The peer-reviewed proceedings of that meeting were subsequently published in *Health Physics* in 1987 (see [25]). This meeting indirectly encouraged a series of research-related activities which greatly promoted the hormesis initiative. Secondly, the rapid transition to *in vitro* toxicology and alternatives to whole animal testing also markedly accelerated in the early to mid-1980s. This led to the evaluation of large numbers of toxic chemicals and drugs on a wide range of biological models in which multiple concentrations could be efficiently and concurrently tested on well plate readers that could accommodate far larger numbers of doses/concentrations than traditional whole animal tests (e.g., 10–11 concentrations using 96-well plate readers as compared to the 2–3 doses used in most whole animal studies). This provided an experimental vehicle to efficiently test agents over a broad range of concentrations, thereby providing a framework to evaluate hormetic concepts and hypotheses.

These activities and the efforts of many other researchers with *in vivo* test protocols have led to the creation of a large hormesis database of many thousands of dose responses, all satisfying rigorous evaluative criteria based on study design, magnitude of stimulation, statistical significance, and reproducibility of findings [26, 27]. A second database was also created in order to establish the frequency of hormesis in the toxicological and pharmacological literature [28–30]. These findings indicated that hormetic dose responses are widespread, very generalizable, being independent of biological model, the endpoint measured and the chemical class or physical stressor studied. The frequency of hormetic responses approached 40% using rigorous a priori entry and evaluative criteria, leading to the suggestion that this frequency may underestimate its actual occurrence.

The hormetic dose–response model was also tested in fair head-to-head comparisons with the threshold dose response to assess which model could better predict responses in the critical below threshold dose zone. In three major tests, the hormetic model far outperformed the threshold model [28, 29, 31–34]. The hormetic model displayed a very good capacity to predict low-dose responses with no known limitations. However, the long-revered threshold model was a disappointing failure in each test. These findings are important because they demonstrated that the model used as the basis of all regulatory agencies dealing with noncarcinogenic agents has a critical failing. It simply did not make accurate predictions where it counts most for the public health, that is, in the low-dose zone.

Quantitative Features of Hormetic Dose Responses

The quantitative features of the hormesis dose response are consistent across all biological models and endpoints measured, thereby making it a very specific type of biphasic dose–response relationship. This is particularly the case with respect to the magnitude and width of the stimulatory responses and the relationship of the stimulatory response to the toxicological threshold [27]. Of importance is that the hormetic stimulation is strikingly and consistently modest, being in the percentage rather than in the fold range. That is, the maximum hormetic stimulation is typically about 30–60% greater than control values, rarely exceeding the control by twofold (Fig. 1). The width of the stimulatory response is usually over a 10- to 20-fold range, although in about 5% of the more than 8,000 cases assessed the width of the stimulation has exceeded some 1,000-fold. The cause of this variability in the width of the stimulation is uncertain but might be related to heterogeneity of the test population. The consistency of the stimulatory response to the toxic threshold is also important since it provides a quantitative linkage to the traditional toxicological threshold and therefore permits the hormesis concept to be consistently integrated into standard risk assessment methods. It also permits all past risk assessments to be reinterpreted within a hormetic context [31, 35–37].

The quantitative features of the hormetic dose response have a number of important implications. First, the modest magnitude of the stimulatory response makes hormesis difficult to prove. This has been a reason for its slow acceptance by the scientific community. The assessment of hormesis-related hypotheses requires the use of more subjects to enhance statistical power calculations because the expected response is modest and control group variation, depending on the biological model, can be an important concern; more doses are also often needed to carefully define the nature of the dose response in the low-dose (i.e., below threshold) zone; multiple temporal evaluations (i.e., repeat sampling activities) are needed to detect the compensatory response [38]; there is also an enhanced need for replication given the modest nature of the low-dose stimulatory effect. Efforts to increase statistical power and to ensure that the results are consistently reproducible are necessary in order to determine whether the hormetic stimulation is “real” and not accounted for by normal background variation. Many investigators are reluctant to design and conduct such rigorous investigations due to the extra time and resources required. This has resulted in dose–response relationships being inadequately designed, especially in the low-dose zone where the greater resources need to be directed.

Investigators often justify the use of only a few high doses since the threshold model assumes that there are no treatment-related effects below the threshold. These assumptions and the practices that gave birth to the threshold model are what resulted in toxicology evolving into a few high-doses discipline, a practice that has led many to question its capacity to offer insights on the critical questions of today that deal with a preponderance of low-dose exposures to complex mixtures.

A second implication of the modest stimulation is that it relates principally to biological performance, not toxicity. The hormetic dose response indicates that chemicals/drugs that induce a low-dose stimulation will be constrained by the

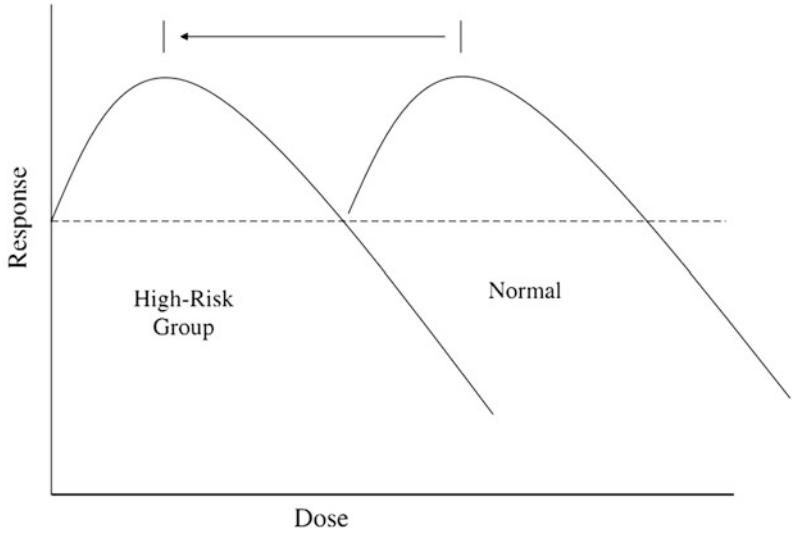


Fig. 2 Stylized dose–response curves illustrating the presence of a hormetic response in high-risk groups. This response occurs at lower doses compared with the response in the normal population and thus the dose–response curve for the high-risk group is shifted to the left of the dose–response curve for the normal population

quantitative features of this dose response [39]. For example, agents that increase cognition, hair growth, longevity, and other performance endpoints are constrained to do so within the 30–60% range of the hormetic dose response. This is the case even when two or more drugs interact in an additive or synergistic fashion [40, 41]. In effect, the concept of synergy in hormesis is far different from that seen in traditional toxicology [42]. In toxicology, the focus is on the magnitude of the response (i.e., toxicity). In hormesis, it is principally on the dose, with the response maxima being constrained. In addition, the hormetic dose–response constraints are seen even when chemicals have profoundly differing potencies. That is, two agents that differ by over a millionfold in toxic potency will nonetheless display the same quantitative features of their respective hormetic responses. The only difference is that the more potent agent will have its dose response shifted far to the left.

The width of the hormetic dose response and its relationship to the toxic threshold are also important factors to consider. The hormetic stimulation is generally fairly close to the toxic threshold, with the maximum hormetic stimulation being within about a factor of 5–10 of the threshold. This has important implications, especially for heterogeneous populations such as with most human groups. Since human interindividual variation can exceed a factor of one hundred [43], it would not be unexpected that what may be a beneficial hormetic response for some may be in the toxic zone for others (Fig. 2). Since target doses are not based on genetic individuality but typically determined by body weight or surface area [44], it is likely that drugs whose beneficial response is in the hormetic zone could have the potential to induce toxicity in the high-risk segments of that

population. On the other hand, if there were a broad hormetic stimulatory zone, the clinician could target the drug dose optima to be further from the toxic zone with the patient still receiving the intended beneficial effects [45].

Hormesis: An Adaptive Response

The hormetic stimulation has been observed to represent an overcompensation to a disruption in homeostasis [46–48]. The hormesis phenomenon is therefore a dose-time response in which there is an initial stress or damage induced by the causative agent. However, the affected biological system responds in a compensatory manner to repair the damage. The compensatory response usually slightly overshoots the original homeostatic set point (i.e., equal to the control group value). This is reflected in the modest stimulation that characterizes the hormetic response in the low-dose region. However, at high doses where toxicity is excessive, a full and successful compensatory response is usually not achieved, and this is reflected in the high-dose toxicity response. The hormetic stimulation response therefore is adaptive in nature and represents a reparative response of the affected biological system. The response to the low-dose induction of damage also affects a series of prosurvival adaptive responses that permits the biological system to be protected against a more massive subsequent exposure to the toxic agent. This has been referred to as an adaptive or conditioning response. This dose response of the "adapting" or the "conditioning" doses to the more massive subsequent treatment follows the inverted U-shape of the hormetic dose response [49].

While hormesis is generally viewed as an adaptive response, there are situations when it can be considered maladaptive. For example, low doses of many antitumor agents can enhance the proliferation on tumor cells. This has now been recognized and under certain circumstances may pose an enhanced risk to the patient [50]. This same type of process could also occur following exposures to antibiotics, antifungal agents, and antiviral medications. It is possible that the low-dose stimulation could be harmful if it enhanced endpoints related to autoimmunity [51]. Likewise, certain drugs, such as ouabain, that have been used to treat congestive heart failure can enhance the proliferation of smooth muscle of the prostate gland by about 30% [52]. This magnitude of enlargement may be sufficient to cause clinical symptoms in affected patients. Thus, the concept of benefit and harm should be decoupled from the definition of hormesis [53] since the implications of the low-dose stimulation could be beneficial, harmful, neutral, or unknown.

Hormesis: An Example of Biological Leveraging

While recognizing the potential adverse effects of the low-dose stimulation and how these may be dealt with in order to enhance healthy outcomes, it is important to

place the hormetic dose responses in a broad context. The hormetic dose response is an adaptive strategy that reflects a type of "biological leveraging." The low-dose exposure induces a stress or low-grade toxicity response. The affected biological system then generates a compensatory response that typically exceeds the original set point condition (i.e., equivalent of the control response) in a modest fashion (i.e., 30–60% at maximum). This response not only repairs the initial modest damage but also provides benefits that significantly outweigh the costs of the initial investment (i.e., the initial stress or toxicity). Hormesis may therefore be seen as an investment strategy that not only protects against status quo stresses (i.e., the induced modest toxicity and background toxicity by about 30%) but also preadapts the affected system(s) against potential catastrophic loss, that is, death or significant disability. The concept of biological leveraging is seen in numerous toxicological and biomedical settings (e.g., adaptive responses to radiation and chemical mutations; preconditioning hypoxic stress and its protection against cardiac injury) [49, 54]. All such situations have an adapting dose or conditioning stress optima which conform to the quantitative features of the hormetic dose response.

Hormesis and Drug Discovery/Development

The hormetic dose response is also being exploited routinely in the pharmaceutical world but usually under the guise of terms such as biphasic, U-shaped, and bell-shaped, that is, interchangeable terms for the same dose–response relationship. For example, detailed evaluations within the field of antianxiety drugs revealed that essentially all the standard animal screening tests (e.g., elevated plus maze, hole board test, light–dark test, four plates test, open field test, staircase test, social interaction test) demonstrate inverted U-shaped dose responses, all consistent with the quantitative features of the hormetic dose response [55]. In such cases, the low-dose stimulation typically reflects the zone of reduced anxiety. Thus, in these instances, the hormetic dose response is that which is used to screen and judge antianxiety drugs and move the effective ones along for further testing.

With respect to antiseizure drugs, animals are employed to assess agents that can modulate chemically induced seizure thresholds. Those agents that can increase the chemically induced threshold dose, that is, making it harder to induce a seizure, may have potential as antiseizure agents. In this case as well, effective antiseizure agents act biphasically in a manner consistent with the hormetic dose response, increasing the threshold at low doses while decreasing the threshold (i.e., making it easier to induce a seizure) at higher doses. This is another case where pharmaceutical companies have long been using the hormetic dose–response concept [56]. In the case of memory and cognitive dysfunction, including those approved for the treatment of Alzheimer's disease [57], all have shown the typical inverted-U of the hormetic dose response, again with the same quantitative features of the dose response. Similar effects have been reported for pain modulation [58], for chemical-induced nausea [58], for protecting neurons from a wide range of

chemical-induced stressors [59], for enhancing neuronal outgrowths [60], for experimentally induced stroke damage/brain traumatic injury [61], and for drug addiction [62].

The neurosciences therefore display a broad and extensive array of hormetic dose responses. Until very recently, the term hormesis only rarely has been used to describe the plethora of hormetic dose responses in this area [54]. The field has typically used nonspecific terms such as biphasic, dual effects, U-shaped, bell-shaped, pharmacological inversion, nonmonotonic, and others for hormetic-like dose–response relationships. However, these various dose responses share the same quantitative features and the same inherent constraints. In effect, this seemingly highly diverse array of biphasic dose–response relationships is all hormetic. This is the case for other biomedical domains, as has been recently shown for immunological responses [50], human tumor cell responses [51], and the vast array of other areas reflected in the hormesis database [27].

Hormesis: Gaining Visibility and Acceptance

It is important to note that the hormetic dose response is being rediscovered by the scientific community or, to be more accurate, discovered for the first time. For example, leading textbooks in human and environmental toxicology now include sections on hormesis [21, 63–65]; entire chapters have been published in textbooks dealing with aquatic toxicity [39] and pharmacology [22], and chapters have been included in a number of monographs [47]. Within the year 2010, three books have been published on hormesis [66–68]. Major professional societies, such as the US Society of Toxicology (SOT), have recently had a major session on hormesis at their annual conference. Even more notable is that leading indexing services such as the Web of Science reveal over 1,500 citations on hormesis, with more than 80% of those since 2000. Despite this positive indicator of growing interest, this number may substantially underestimate/hormetic dose responses, due to the use of alternative terms, among other factors. Hormesis has also been the object of numerous detailed stories reaching the general public as seen in substantial articles in *The Wall Street Journal* [69], *U.S. News & World Report* [70], *Discover Magazine* [71], *The Boston Globe* [72], *The Baltimore Sun* [73], *Science News* [74], as well as a four-page detailed story in the news section of *Science* [75].

Summary

The hormesis story is important for a number of reasons. First, it has revealed that the historical foundations leading to the acceptance of the threshold dose–response model throughout the twentieth century and even down to today were based on political and economic concerns rather than science. This analysis also reveals what

may be hard to believe and even more difficult to accept. That the field of toxicology and the biomedical sciences in general accepted the threshold dose–response model without validation of its capacity to predict responses in the low-dose zone (i.e., below the toxic/pharmacological threshold) and then continued to apply this unproven model for that purpose for at least the past six decades, jeopardizing the public health while thinking it was doing what was scientifically correct. That an entire field could make a mistake on the central pillar of its discipline and perpetuate that error for generations of scientists is as remarkable as it is disconcerting. Despite the historical blunders that have guided toxicology and regulatory agencies on the nature of the dose response, the hormesis story provides a key framework for guiding the scientific community in the development of a new generation of pharmaceutical agents and chemically based products that can better exploit the nature of the dose response to enhance human and environmental health, respectively, including more protective and scientifically based health standards, to enhance biological efficiencies in a broad range of systems of economic and health benefit, to improve cost-benefit assessments, and to better understand the limitations of biological systems due to plasticity restrictions in the development of pharmaceuticals. Hormesis can provide insight in addictive behaviors as self-administration of addictive drugs often follows the quantitative features of the hormetic dose response [60]. Finally, the emerging evidence indicates that there is a healthy side of stress that can and should be exploited for personal and societal gain. However, the dose response must be both better understood and respected because by its very nature it is biphasic, and these phases can be in close proximity, with health and disease being close neighbors.

References

1. Shackell LF (1925) The relation of dosage to effect. II. J Pharmacol Exp Ther 15:275–288
2. Shackell LF, Williamson W, Deitchmann MM, Katzman GM, Kleinman BS (1924/1925) The relation of dosage to effect. J Pharmacol Exp Ther 23/24:53–65
3. Clark AJ (1933) Mode of action of drugs on cells. Arnold, London
4. Clark AJ (1937) Handbook of experimental pharmacology. Springer, Berlin
5. Calabrese EJ (2005) Historical blunders: how toxicology got the dose–response relationship half right. Cell Mol Biol 51:643–654
6. Calabrese EJ (2010) Hormesis is central to toxicology, pharmacology and risk assessment. Hum Exp Toxicol 29:249–261
7. Calabrese EJ, Baldwin LA (2003) Toxicology rethinks its central belief—hormesis demands a reappraisal of the way risks are assessed. Nature 421:691–692
8. Southam CM, Ehrlich J (1943) Effects of extracts of western red-cedar heartwood on certain wood-decaying fungi in culture. Phytopathology 33:517–524
9. Calabrese EJ (2009) The road to linearity: why linearity at low doses became the basis for carcinogen risk assessment. Arch Toxicol 83:203–225
10. Calabrese EJ (2009) Getting the dose–response wrong: why hormesis became marginalized and the threshold model accepted. Arch Toxicol 83:227–247
11. Schulz H (1887) Zur Lehre von der Arzneiwirkung. Virchows Arch Pathol Anat Physiol Klin Med 108:423–445
12. Schulz H (1888) Über Hefegifte. Pflugers Arch Gesamte Physiol Menschen Tiere 42:517–541

13. Böhme H (1986) Hugo Schulz (6.8.1853–13.7.1932)—sein Leben und Werk. M.D. Thesis, Medical Department, Free University of Berlin, Germany
14. Crump T (2003) NIH library translation (NIH-98-134). Contemporary medicine as presented by its practitioners themselves, Hugo Schulz, Leipzig, 1923: 217–250. Nonlinearity Biol Toxicol Med 1:295–318
15. Coulter HL (1982) Divided legacy: the conflict between homeopathy and the American Medical Association. North Atlantic Books, Richmond, CA
16. Calabrese EJ, Baldwin LA (2000) Chemical hormesis: its historical foundations as a biological hypothesis. Hum Exp Toxicol 19:2–31
17. Calabrese EJ, Baldwin LA (2000) The marginalization of hormesis. Hum Exp Toxicol 19: 32–40
18. Calabrese EJ, Baldwin LA (2000) Radiation hormesis: its historical foundations as a biological hypothesis. Hum Exp Toxicol 19:41–75
19. Calabrese EJ, Baldwin LA (2000) Radiation hormesis: the demise of a legitimate hypothesis. Hum Exp Toxicol 19:76–84
20. Calabrese EJ, Baldwin LA (2000) Tales of two similar hypotheses: the rise and fall of chemical and radiation hormesis. Hum Exp Toxicol 19:85–97
21. Calabrese EJ (2008) Hormetic vignette—the dose response: comparing hormesis and threshold models. In: Newman MC (ed) Fundamentals of ecotoxicology, 3rd edn. CRC Press, Boca Raton, FL
22. Calabrese EJ (2009) Hormesis as a basic concept. In: Hacker M, Bachmann K, Messer W (eds) Pharmacology: principles and practice. Elsevier, Oxford, pp 75–102
23. NCRPM (1954) Permissible dose from external sources of ionizing radiation, vol 17, U.S. National bureau of standards handbook 59NCRP Rep. U.S. Department of Commerce, National Council on Radiation Protection and Measurements, Washington, DC
24. NAS (1977) Drinking water and health. National Academy Sciences, Washington, DC
25. Calabrese EJ, McCarthy M, Kenyon E (1987) The occurrence of chemical hormesis. Health Phys 57:531–542
26. Calabrese EJ, Baldwin L (1997) The dose determines the stimulation (and poison): development of a chemical hormesis database. Int J Toxicol 16:545–559
27. Calabrese EJ, Blain R (2005) The occurrence of hormetic dose responses in the toxicological literature, the hormesis database: an overview. Toxicol Appl Pharmacol 202:289–301
28. Calabrese EJ, Baldwin LA (2001) The frequency of U-shaped dose–responses in the toxicological literature. Toxicol Sci 62:330–338
29. Calabrese EJ, Baldwin LA (2003) The hormetic dose response model is more common than the threshold model in toxicology. Toxicol Sci 71:246–250
30. Calabrese EJ, Blain RB (2009) Hormesis and plant biology. Environ Pollut 157:42–48
31. Calabrese EJ (2008) Hormesis: why it is important to toxicology and toxicologists. Environ Toxicol Chem 27:1451–1474
32. Calabrese EJ, Staudenmayer JW, Stanek EJ, Hoffmann GR (2006) Hormesis outperforms threshold model in NCI anti-tumor drug screening data. Toxicol Sci 94:368–378
33. Calabrese EJ, Stanek EJ, Nascarella MA, Hoffmann GR (2008) Hormesis predicts low-dose responses better than threshold models. Int J Toxicol 27:369–378
34. Calabrese EJ, Hoffmann GR, Stanek EJ, Nascarella M (2010) Hormesis in high-throughput screening of antibacterial compounds in *E coli*. Hum Exp Toxicol 29:667–677
35. Calabrese EJ, Cook RR (2005) Hormesis: how it could affect the risk assessment process. Hum Exp Toxicol 24:265–270
36. Cook RR, Calabrese EJ (2006) The importance of hormesis to public health. Environ Health Perspect 114:1631–1635
37. Cook RR, Calabrese EJ (2006) Hormesis is biology, not religion. Environ Health Perspect 114:A688
38. Calabrese EJ (2001) Overcompensation stimulation: a mechanism for hormetic effects. Crit Rev Toxicol 31:425–470

39. Calabrese EJ (2012) Hormesis: the most common and fundamental dose response relationship in toxicology. In: Rand GM, Lewis MA, Klaine SJ (eds) Fundamentals of aquatic toxicology: effects, environmental fate and risk assessment, 3rd edn. CRC Press, Boca Raton, FL
40. Flood JF, Smith GE, Cherkin A (1983) Memory retention: potentiation of cholinergic drug combinations in mice. Neurobiol Aging 4:37–43
41. Flood JF, Smith GE, Cherkin A (1985) Memory enhancement: supra-additive effect of subcutaneous cholinergic drug combinations in mice. Psychopharmacology 86:61–67
42. Calabrese EJ (2008) Hormesis and mixtures. Toxicol Appl Pharmacol 229:262–263
43. Calabrese EJ (1985) Uncertainty factors and interindividual variation. Regul Toxicol Pharmacol 5:190–196
44. Calabrese EJ (1983) Principles of animal extrapolation. Wiley, New York, NY
45. Calabrese EJ (2007) A dose of common sense. Good Clin Pract J July:12–16
46. Calabrese EJ (1999) Evidence that hormesis represents an "overcompensation" response to a disruption in homeostasis. Ecotoxicol Environ Saf 42:135–137
47. Calabrese EJ (2008) What is hormesis? In: Le Bourg E, Rattan SIS (eds) Mild stress and healthy aging: applying hormesis in aging research and interventions. Springer, Berlin, pp 5–19
48. Calabrese EJ, Baldwin LA (2002) Applications of hormesis in toxicology, risk assessment and chemotherapeutics. Trends Pharmacol Sci 23:331–337
49. Calabrese EJ (2008) Converging concepts: adaptive response, preconditioning, and the Yerkes–Dodson Law are manifestations of hormesis. Ageing Res Rev 7:8–20
50. Calabrese EJ (2005) Cancer biology and hormesis: human tumor cell lines commonly display hormetic (biphasic) dose responses. Crit Rev Toxicol 35:463–582
51. Calabrese EJ (2005) Hormetic dose–response relationships in immunology: occurrence, quantitative features of the dose–response, mechanistic foundations and clinical implications. Crit Rev Toxicol 35:89–306
52. Qiu J, Gao HQ, Zhou RH, Liang Y, Zhang XH, Wang XP, You BA, Cheng M (2007) Proteomics analysis of the proliferative effect of low-dose ouabain on human endothelial cells. Biol Pharm Bull 30:247–253
53. Calabrese EJ, Baldwin LA (2002) Defining hormesis. Hum Exp Toxicol 21:91–97
54. Calabrese EJ, Bachmann KA, Bailer AJ, Bolger PM, Borak J, Cai L, Cedergreen N, Cherian MG, Chiueh CC, Clarkson TW *et al* (2007) Biological stress response terminology: integrating the concepts of adaptive response and preconditioning stress within a hormetic dose–response framework. Toxicol Appl Pharmacol 222:122–128
55. Calabrese EJ (2008) An assessment of anxiolytic drug screening tests: hormetic dose responses predominate. Crit Rev Toxicol 38:489–542
56. Calabrese EJ (2008) Modulation of the epileptic seizure threshold: implications of biphasic dose responses. Crit Rev Toxicol 38:543–556
57. Calabrese EJ (2008) Alzheimer's disease drugs: an application of the hormetic dose response model. Crit Rev Toxicol 38:419–451
58. Calabrese EJ (2008) Pain and U-shaped dose responses: occurrence, mechanisms and clinical implications. Crit Rev Toxicol 38:579–590
59. Calabrese EJ (2008) Pharmacological enhancement of neuronal survival. Crit Rev Toxicol 38: 349–384
60. Calabrese EJ (2008) Enhancing and regulating neurite outgrowth. Crit Rev Toxicol 38: 391–418
61. Calabrese EJ (2008) Drug therapies for stroke and traumatic brain injury often display U-shaped dose responses: occurrence, mechanism and clinical implications. Crit Rev Toxicol 38:557–577
62. Calabrese EJ (2008) Addiction and dose response: the psychomotor stimulant theory of addiction reveals that hormetic dose responses are dominant. Crit Rev Toxicol 38:599–617
63. Beck B, Calabrese EJ, Slayton TM, Rudel R (2007) The use of toxicology in the regulatory process. In: Hayes AW (ed) Principles and methods of toxicology, 5th edn. CRC Press, Boca Raton, FL, pp 45–102

64. Beck BD, Slayton TM, Calabrese EJ, Baldwin L, Rudel R (2001) The use of toxicology in the regulatory process. In: Hayes AW (ed) Principles and methods of toxicology, 4th edn. Taylor & Francis, Philadelphia, PA, pp 23–76
65. Eaton DL, Klaassen CD (2003) Principles of toxicology. In: Klaassen CD, Watkins JB III (eds) Casarett and Doull's essentials of toxicology. McGraw-Hill, New York, NY, pp 6–20
66. Mattson MP, Calabrese EJ (2010) Hormesis: a revolution in biology, toxicology and Medicine. Humana, New York, NY
67. Sander CL (2010) Radiation hormesis and the linear-no-threshold assumption. Springer, Berlin
68. Elliott KC (2010) Is a little pollution good for You? incorporating societal values in environmental research. Oxford University Press, New York, NY
69. Begley S (2003) Scientists revisit idea that a little poison could be beneficial. Wall St J CCXLII(121):104
70. Boyce N (2004) Is there a tonic in the toxin? U.S. News & World Report, 18 Oct 2004, 137 (13):74–75
71. Hively W (2002) Is radiation good for you? Discover Magazine 23:75–80
72. Cook G (2003) A scientist finds benefit in small doses of toxins. The Boston Globe, 12 Dec 2003, 264(165):A1 and A16
73. Bell J (2004) A little poison, a big help? The Baltimore Sun, 15 Mar 2004
74. Raloof J (2007) Counterintuitive toxicology. Sci News 171:40–42
75. Kaiser J (2003) Sipping from a poisoned chalice. Science 302:376–379

Index

A
AAS. *See* Anabolic-androgenic steroid (AAS)
Aberrant DNA methylation, 507, 526
Accurate mass, 477, 479
ACD. *See* Allergic contact dermatitis (ACD)
Acetaminophen (APAP), 529
2-Acetylaminofluoren (2-AAF), 511, 512, 529, 530
Acetylation, 500, 502, 513, 515, 517, 518, 525, 526
Activator protein 1 (AP-1), 179, 180, 192, 193
Acute promyelocytic leukemia, 136, 139
Adenocarcinomas, 142
Adherens junctions (AJ), 329
Adjuvant, 174, 193
Agglomerates, 224, 240
Aggregates, 224, 228
Agouti gene, 508
Agouti mouse model, 519
AHR. *See* Aryl hydrocarbon receptor (AHR)
AHR signaling pathway, 533
AIS. *See* Androgen insensitivity syndrome (AIS)
Alcohol dehydrogenase (ADH), 442, 443
  and HDL-cholesterol, 443
Alcoholic liver disease (ALD), 532
Aldo-keto reductases (AKRs), 115
Alkaptonuria, 438, 440
Allergic airway sensitisation, 193
Allergic contact dermatitis (ACD), 289, 293, 294
Allergic diseases, 290
Allergic inflammation, 121
Allergy, 442
Aluminum (Al), 135, 511, 518
Alzheimer, 178, 179
Alzheimer's disease, 484, 518
Amyloid, 178
Anabolic-androgenic steroid (AAS), 335, 336
Androgen insensitivity syndrome (AIS), 320, 321
Anthracosis, 524
Anthropogenic activities, 134, 136, 143, 146
Anti-benzo[*a*]pyrene-7,8-diol-9,10-epoxide (BPDE), 512, 533, 534
Antibody (antibodies)
  autoantibodies, 258, 259, 269, 271, 275, 276
  cytophilic antibodies, 264–266, 276
  IgA, 254, 269
  IgD, 254
  IgE, 254, 264–266
  IgG, 254, 264, 265
  IgM, 254, 259
  response, 254, 255, 259, 265, 275
  titer, 256, 266, 271
Antigen, 252–255, 257, 259–261, 264, 266–268, 275, 276
  self antigen, 257, 268, 271
Antimicrobial nanosilver, 230
Antinomy (Sb), 135
AP-1. *See* Activator protein 1 (AP-1)
Apoptosis, 118–122, 135, 136, 139, 148
Applicability domains (AD), 388
Apurinic sites, 109, 115
Aquatic toxicity, 4, 394–398, 404
Area under the curve (AUC), 474, 475
Aroclor 1248, 342
Arsenic (As), 134–140, 142, 152, 153, 508, 511, 514–516, 518, 521, 525
Aryl hydrocarbon receptor (AHR), 110, 117, 118, 121, 122, 516, 533
Ascorbic acid, 139, 145
Asthma, 171, 172, 174, 182, 185, 193, 194, 290, 301, 302, 304, 442, 444

A. Luch (ed.), *Molecular, Clinical and Environmental Toxicology*, Experientia Supplementum 101, DOI 10.1007/978-3-7643-8340-4,

Atherosclerosis, 175, 176, 179, 182, 190, 196
Atrazine, 416, 424
AUC. *See* Area under the curve (AUC)
Autoxidation, 115
5-Aza-2′-deoxycytidine, 511, 522, 531

B
Barium (Ba), 135
Barker hypothesis, 316
Base excision repair (BER), 116, 117
BDNF. *See* Brain-derived neurotrophic factor (BDNF)
Benchmark dose (BMD), 262, 263, 277
Benz[*a*]anthracene (BA), 430
Benzo[*a*]pyrene (BP), 108–112, 114–121, 427, 441, 442, 516, 533–534
Benzylic ester pathway, 115
Beryllium (Be), 135
Beverages, 98–99
Bioaccumulation/bioconcentration, 4, 488, 489
Bioinformatics, 440, 441, 448, 452, 462, 466, 467
Biological sensors, 478
Biomarkers, 292, 296, 298–299
Biomonitoring, 475, 478, 487–490
Biotinylation, 502, 517
Bismuth (Bi), 135
Bisphenol A (BPA), 346, 349, 350, 511, 512, 519–522
Bisulfite sequencing, 519
Blank problem, 102, 103
Bleomycin, 521
Blood-testis-barrier (BTB), 319, 329, 331, 350
B lymphocyte/B cell, 254, 269, 274
BMD. *See* Benchmark dose (BMD)
BP. *See* Benzo[*a*]pyrene (BP)
BPA. *See* Bisphenol A (BPA)
Brain-derived neurotrophic factor (BDNF), 511, 512, 517, 518, 532
Breastfeeding, 90
Bronchitis, 171, 172
Bulky DNA adduct, 107–122

C
Cadmium (Cd), 135, 140–142, 152, 153, 517–518, 525
Carbendazim (CBZ), 334, 335, 342
Carbon black, 168, 169, 174, 182, 185–188, 191–195, 524
Carbon nanotubes (CNTs), 225, 230, 236, 237
Carbon tetrachloride (CCl_4), 511, 512, 529
Carcinogenesis, 135, 138, 140, 142, 145, 146, 151, 152
Carcinogenicity, 4, 135, 137–142, 144–146, 148–152, 389, 399–400
Carcinogen, 135, 138, 142, 143, 149
Cardboard, 101
Cardiovascular disease, 171, 175, 182, 187
Causality and biases
  confounding, 451
  Mendelian randomization, 451
  reverse causality, 451
CBZ. *See* Carbendazim (CBZ)
Cell-based assay, 42
Cell cycle
  arrest, 118, 119
  checkpoints, 118, 119
  progression, 115, 116, 120
Cell lines, 292, 295, 296, 298, 301–303
Cell surface markers, 296
Chemical emergencies, 12, 16–17
Chemical induced damage to the environment, 18
Chemical induced injury and disease, 18
Chemical mixtures, 342, 370–371
Chemical safety
  management system, 8, 9, 11, 18
  policy, 8–11, 18
  procedures, 15
  strategic plan, 8, 11, 18
  work instructions, 10
Chemical space, 477
Chemicals storage, 5, 8, 11, 12, 15–17, 19
Chemicals substitution, 14
Chemicals transport, 5, 11, 15–16
Chemical toxicity, 381–405
Cheminformatics, 383, 389, 391, 393, 394, 398, 402, 404
Chemistry of the chirality, 414–415
Children, 146–148
Chlordane, 416–418, 420, 424
Chloroborane, 416
Chromium (Cr), 134, 135, 142–146, 152, 515–517, 525
Chronic degenerative diseases, 526
Chronic obstructive pulmonary disease (COPD), 171, 172, 182, 193
Cigarette smoke, 511, 526–528
Clark, Alfred J.
  criticism of Schulz, 553
CNTs. *See* Carbon nanotubes (CNTs)
Cobalt (Co), 134
Collagen, 172, 193
Combustion-derived particles, 166, 168
Combustion processes, 109
Commercial toxicity databases, 389

Comparative Toxicogenomics Database (CTD), 447
Complex metabolomic platforms, 481–482
Complex mixtures, 109, 115
Composition, 167, 168, 174
Computational modeling, 460, 462, 466
Concentrated ambient particles (CAP), 168, 169, 175, 176, 179, 184, 196
Congeners, 109
Congenital adrenal hyperplasia (CAH), 321
Consensus model, 388, 394–398, 404
Consumer products, 229–230
COPD. *See* Chronic obstructive pulmonary disease (COPD)
Copper (Cu), 134, 135, 152
Corrosives, 2, 3
COX-2. *See* Cyclo-oxygenase-2 (COX-2)
Creatine kinase (CK), 473
Cryptorchidism, 320, 324–326, 336, 345, 346, 350
Cyclodiene pesticides, 420
Cyclo-oxygenase-2 (COX-2), 178, 183, 187, 188
CYP1A1, 110, 111, 122
CYP1B1, 110
Cytochrome P450-dependent monooxygenase (CYP), 109, 111, 112, 114, 115, 122
Cytokine, 294–296, 298

D
Dangerous goods, 2–4, 15
Dangerous when wet substances, 3
Database, 383, 384, 389–394, 401, 403
DBCP. *See* 1,2-Dibromo-3-chloropropane (DBCP)
Degradability, 4
Dendritic cell (DC), 174, 185, 186, 189, 193–195, 292, 294–299, 301–303
Depurination, 115
DES. *See* Diethylstilbestrol (DES)
Desert hedgehog (DHH), 319
Detoxification, 135, 138, 145
Developmental toxicity, 52, 56–58, 64, 65
Diabetes, 171, 175
Dibenzo[*a,l*]pyrene (DBP), 108, 110–112
Dibenzopyrenes, 114
1,2-Dibromo-3-chloropropane (DBCP), 333, 334
Dichlorprop, 416, 417
Diesel engine exhaust, 169, 174, 175, 179
Diesel exhaust, 524, 525
Diesel exhaust particles (DEP), 168, 169, 172, 174, 176, 185–189
Diethylstilbestrol (DES), 324, 346, 349, 511, 512, 520–522
Di-(n-butyl) phthalate (DBP), 326, 327, 341
Diol-epoxide, 109, 111, 112, 114, 115
Diol-epoxide activation pathway, 110–114, 122
Dioxins, 516, 533–534
Diseases status, 476
Dispersion glue, 101
Dissolved gases, 3
Distribution of chiral pollutants, 414, 416–418, 431
DLVO theory, 228
3D models, 298, 303–304
DNA adducts, 442
DNA damage, 109–121, 135, 142, 148, 151, 152, 180, 181, 191
DNA methylation, 316, 338, 339, 344, 345, 500–502, 506–510, 513–515, 517, 518, 520, 522, 524, 526, 529
DNA methyltransferases (DNMTs), 501, 503, 507, 508, 514, 517, 522, 523, 526
DNA repair, 112, 115–120, 122
Dose-response
  threshold dose-response, 552, 555, 560, 561
Duty of care, 5
Dynamic light scattering (DLS), 234

E
Ecotoxics, 2
EDS. *See* Ethylene-1,2-dimethanesulfonate (EDS)
Ehrlich, John
  named hormesis, 552
Emphysema, 141
Endocrine-disrupting chemicals (EDCs), 322–325, 327, 329, 344–350, 518, 519, 521, 523, 524
Endocrine disruption, 54, 60–64
Endocrine disruptors, 518–524
Endothelial, 172, 175, 178, 184, 195–196
Endothelial cells, 172, 184, 185, 192, 195–197
Endotoxin, 171, 174, 181, 190, 192, 195
Engineering controls, 14
Envirome, 440, 450–452
Environmental cigarette smoke, 527
Environmental exposure, 50–51
Environmental Protection Agency (EPA)
  environmental health standards, 552
Environmental Specimen Bank (ESB), 488
Environment-wide association study (EWAS), 450, 451
Enzyme inducible nitric oxide synthase (iNOS), 181, 183
Epidemiology, 65–67
Epididymis, 317–320, 324, 329, 331, 332
Epigenetics, 439, 450, 451
  and exposure, 439, 450

Epithelial, 292, 295, 302–304
Epithelial cells, 167, 172, 181, 182, 184–186, 188, 191–195, 197
ESB. *See* Environmental Specimen Bank (ESB)
Estrogen responsive element (ERE), 520
Ethanol, 512, 532–533
Ethylene-1,2-dimethanesulfonate (EDS), 334, 335, 337
Euchromatin, 502, 513
EWAS. *See* Environment-wide association study (EWAS)
Explosive, 3, 17
Exposure, 134–139, 141–144, 146–153
Exposure routes, 238–240
Expression quantitative trait locus (eQTL), 446
Extrapulmonary translocation, 178

F
Fast liquid chromatography (FAST LC), 477, 482
Fate and transport, 49–50
Fenton reaction, 113
Fetal alcohol syndrome, 512, 532
Fibroblasts, 172, 185, 193, 197
Fibrosis, 172
Fish
  consumption, 150
  products, 97–98
Flammable gases, 3
Flammable liquids, 3
Flammable solids, 3
5-Fluorouracil (5-FU), 511, 512, 531
Folate-deficient diet, 508, 510
Food processing, 99–101

G
Gallium (Ga), 135
Garrod, Archibald, 438, 440
Gas, 3, 17
Gases under pressure, 3
Gaskets, 101, 102
GDNF. *See* Glial cell line-derived neurotrophic factor (GDNF)
Gene, 438–447, 450–452
  gene-environment interaction, 438, 444–446, 448, 449, 451, 452
Gene Expression Omnibus (GEO), 447
Generations of nanomaterials, 226
Genetic imprinting, 343
Genistein, 519
Genome-wide association study (GWAS), 440, 445, 446, 448–451
  and nicotine, 446
Genome-wide interaction study (GEWIS), 446
Genomic biomarkers, 461–462
Genotoxicity, 4, 113, 115, 135, 138, 139, 149
German Environmental Survey for Children (GerES IV), 488
Germanium (Ge), 135
Glial cell line-derived neurotrophic factor (GDNF), 347
Global genome repair (GG), 116
Globally harmonized systems for classification and labelling of hazardous chemicals (GHS), 2–5
Glutamic oxaloacetic transaminase, 473
Glutathione, 142, 145, 148, 151, 152
Gonadotropin releasing hormone (GnRH), 321, 328, 331, 336, 337, 339, 340, 350
Gonadotropin secretion, 328, 331
Grain, 98, 101
Granulocyte macrophage colony-stimulating factor (GM-CSF), 183, 187, 188, 191, 193, 195
GWAS. *See* Genome-wide association study (GWAS)

H
Hard corona, 233
HATs. *See* Histone acetyltransferases (HATs)
Hazard, 292, 295, 302, 304
Hazard communication, 3–5, 7, 8, 11, 12
Hazardous chemicals, 2, 5–19
Hazardous substances, 2
HCG. *See* Human chorionic gonadotropin (HCG)
HDACs. *See* Histone deacetylases (HDACs)
Health effects, 137, 145, 152
Health surveillance, 7, 11, 16
Healthy status, 476
Heart attacks, 174
Heart rhythm disturbances, 174
Heavy metals, 133–153
Heme oxygenase-1 (HO-1), 173, 180, 195
Hepatic transcript index (HTI), 41–42
Hepatocarcinogenesis, 511, 512, 529, 530
Hepatotoxicants, 529
Hepatotoxicity, 54–56, 64, 151
Heterochromatin, 502–504, 506, 513, 529
2,5-Hexanedione (HD), 333, 334, 337, 342
Hexogen (RDX), 511, 512, 531–532
High-density polyethylene (HDPE), 95
High-throughput analysis, 477, 480, 482
High-throughput metabolic fingerprinting, 477
High throughput screening (HTS), 385, 390, 398–400
Histone acetylation, 515, 517

Histone acetyltransferases (HATs), 501, 502, 516
Histone deacetylases (HDACs), 501–503, 515–517, 522, 528
Histone demethylases, 514
Histone methyltransferases (HMTs), 501–503, 520
Histone modification, 501–503, 506, 513, 515–518, 525, 526
Homeopathy
  relationship to hormesis, 553
Homeostasis, 150, 151
Hormesis, 342
  database, 555, 560
  first hormesis conference, 555
  historical foundations, 552, 553, 560
  name, 552–554
  quantitative features, 553, 554, 556–561
  resurgence in toxicology, 554–555
HTI. *See* Hepatic transcript index (HTI)
Human biomonitoring, 475, 478, 487–490
Human chorionic gonadotropin (HCG), 319, 472
Human estrogen receptor (hER), 421, 423
Human exposure, 134–137, 141, 143–144, 147, 150, 153
Human milk, 90–94
Hybrid chemical-biological descriptors, 399–400
Hydrogen peroxide (H_2O_2), 113–115, 121
8-Hydroxy-2′-deoxyguanosine (8-OH-dG), 113, 181, 182, 191, 525
Hydroxyl radicals (•OH), 113, 181
Hypospadias, 320, 324, 326, 341, 346, 350
Hypothalamic hypogonadism, 321
Hypothalamic-pitutary-adrenocortical (HPA), 450
Hypothalamus-pituitary-gonadal (HPG) axis, 328, 329, 336, 339, 349, 350

I
IARC. *See* International Agency for Research on Cancer (IARC)
IC_{50}, 384, 401, 402
ICAM-1. *See* Intercellular adhesion molecule-1 (ICAM-1)
Identification, 290–293, 296, 297, 301, 303, 304
Ifosfamide, 430
IL-6, 165, 175, 177, 183, 187, 188, 190, 191, 194, 195
IL-8, 167, 182, 183, 187–193
IL-1, 179, 187, 191
IL-1, 179, 182, 183, 187, 188, 190, 192, 195
Immunotoxicity, 54, 58–60, 64, 65
Imprinting, 501, 503, 508, 523
In chemico, 290, 291, 293, 304
Individual metabolite patterns, 485
Infant formulae, 96–97
Inflammation, 122, 254, 255, 257, 258, 260, 261, 280, 442, 446
Inflammatory, 167, 172, 174, 177–179, 181–184, 186–189, 195, 197
Inflammatory atherosclerosis, 121
Inflammatory cellular signalling, 121–122
Inflammatory diseases, 483
Ingestion, 238, 239
Inhalation, 238–240
In silico, 290, 291, 293, 304, 382, 383
Intelligence quotient (IQ), 148
Intercellular adhesion molecule-1 (ICAM-1), 175, 176, 183, 190, 192, 193, 195
Inter-individual difference, 448
International Agency for Research on Cancer (IARC), 135, 142, 149
Interspecies differences, 364–369
Inventory control, 11, 12, 19
In vitro, 290–293, 295–304
In vitro fertilization, 479, 480
IQ. *See* Intelligence quotient (IQ)
Iron (Fe), 134, 152
Irritants, 4, 295, 296, 302

J
JNK/SAPK, 121

K
Klinefelter syndrome, 321
K nearest neighbor (kNN), 399–402

L
Labels for containers of chemicals, 3, 4, 7, 12
Lab-on-a-chip, 480
Lactate dehydrogenase (LDH), 473
Lactoferrin gene, 521, 522
Large-scale population studies, 488
LC-MS/MS-based lipidomics, 482
LD_{50}, 389, 401, 402
L-dopa, 431
Lead (Pb), 135, 146–149, 152
Leydig cells, 319, 324, 325, 327–331, 334–337, 339, 340, 346, 350
LH. *See* Luteinizing hormone (LH)
Lifestyle-related diseases, 483
Lipidomics, 482–487
Liquid, 3

Lithium, 511, 528
Local lymph node assay (LLNA), 262, 263, 276
Low-dose response, 342
Lowest-observed-adverse-effect-level (LOAEL), 278
Lowest-observed-effect-level (LOEL), 262, 263
Low-level quantification approach, 476
Lung epithelial cells, 181, 182, 184, 185, 191–193, 195
Lung sensitization, 301–304
Luteinizing hormone (LH), 319, 321, 328, 331, 334–337, 339, 340, 349, 350

M
Macrophage inflammatory protein-2 (MIP-2), 177, 182, 183
Macrophages, 176, 177, 181–194, 196, 197, 292, 301–304
Magnesium (Mg), 134
Malathion, 417, 424
Malondialdehyde, 145, 148
Manganese (Mn), 134
MAPK. *See* Mitogen-activated protein kinase (MAPK)
Marker genes, 299, 302, 303
Mechanism-based, 290, 291
Mechanisms, 135, 137–142, 144–146, 148–153
Mendelian, 440, 449, 451, 452
Mercury (Hg), 135, 149–152
Metabolic variation, 363
Metabolomic biomarkers, 460, 463–464
Metabolomics, 475–482, 484, 486, 487, 490
Metalloids, 134, 152
Metallothioneins, 142
Metallotoxins, 513–518
Methamidophos, 417, 423
Methoxychlor, 522–524
Methylation, 138, 140, 149, 501–503, 506, 508, 509, 511, 513–527, 529
Methylation-sensitive restriction fingerprint (MSRF), 519, 521, 523
5-Methylcytosine DNA glycosylase, 502
Methyl-deficient diet, 509
Methylenenetetrahydrofolate reductase (MTHFR), 442, 443
Methylmercury (MeHg), 517–518
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), 511, 512, 527, 528
Microcapillary electrophoresis, 480
Microglial cells, 186, 196
MicroRNAs (miRNAs), 500, 503–534
Milk products, 94–96
MIP-2. *See* Macrophage inflammatory protein-2 (MIP-2)
MiRNA profiling, 509, 510, 519, 528, 531, 534
Mismatch repair (MMR), 116–118
Mitogen-activated protein kinase (MAPK), 180, 191–193, 195, 196
Mixtures, 152, 153, 462, 464–465, 467
MLR. *See* Multiple linear regression (MLR)
MMR. *See* Mismatch repair (MMR)
Modes of action, 58, 64–65
Molecular biomarkers, 472
Molecular Operating Environment (MOE), 392
Molecular responses, 361–374
Molybdenum (Mo), 134
Monitoring, 7, 11, 14, 16
Monocytes, 175, 176, 182, 185–192, 194–196
MPO. *See* Myeloperoxidase (MPO)
MSRF. *See* Methylation-sensitive restriction fingerprint (MSRF)
MTHFR. *See* Methylenenetetrahydrofolate reductase (MTHFR)
Mullerian duct, 318, 319
Multigenerational animal testing, 316
Multiple linear regression (MLR), 384
Multiple reaction monitoring (MRM), 477
Multiple sclerosis, 178, 179
Multivariate risk prediction model, 474
Multivariate statistical analysis, 475, 481
Mutagens, 138, 142
Myeloperoxidase (MPO), 173, 181

N
N-acetyl cysteine, 139
NADPH oxidase (NOX), 176, 181, 195, 196
Nanoaerosols, 229
Nanoanalytics, 221, 231–236
Nano-bio interface, 233, 234
Nanocomposites, 223, 226, 230
Nanoemulsions, 229
Nanofibers, 223
Nanogold, 225, 227, 228, 239, 240
Nano-HPLC, 480
Nanomaterials, 465
Nanomedicine, 222, 229, 239
Nanoobjects, 223–225, 229
Nanoparticles, 167, 169, 175, 178, 179, 220–242
Nanoparticle space, 231
Nanoplates, 223
Nanorods, 223
Nanosensors, 231
Nanosilver, 225, 228, 230, 232, 239, 241

Nanostructured materials, 223
Nanotubes, 223, 226
Nanowires, 223
National Health and Nutrition Examination surveys (NHANES), 147
Natural killer (NK) cell, 253, 259, 270
Nephrotoxicity, 150, 151
Neuronal cells, 196
Neurotoxicity, 54, 63–64
Nickel (Ni), 134, 135, 142, 508, 513–514, 516, 525
Nitric oxide (•NO), 169, 181, 189, 196
No-observed-adverse-effect-level (NOAEL), 277, 278
No-observed-effect-level (NOEL), 262–264, 266
NOX. *See* NADPH oxidase (NOX)
Nuclear factor κB (NF-κB), 179, 180, 182–185, 188, 191–193, 195
Nucleotide excision repair (NER), 116, 117, 119
Nutriceuticals, 486, 487

O
Occupational health, safety and environment (OHSE), 2, 5, 6, 8, 9, 12
Occupational Safety and Health Administration (OSHA)
  industrial health exposure standards, 552
One-electron oxidation, 109, 114
OpenTOX project, 394, 403
Organic carbon compounds, 168
Origin of chirality, 414
Osteoporosis, 141
Out-of-plane distortions, 109
Oxidative damage, 145, 149, 150
Oxidative genotoxic stress, 509
Oxidative phosphorylation, 138
Oxidative stress, 134, 135, 138–140, 142, 146, 148, 150–152, 175, 176, 178–184, 191–197
Oxidisers, 3
Oxidising peroxides, 3

P
p53, 113, 139, 140
PAH-DNA adduct, 107–122
PAH-DNA damage response, 118–121
PAH-induced tumorigenesis, 110, 113, 115
PAH radical cation, 109, 114, 115
PAHs. *See* Polycyclic aromatic hydrocarbons (PAHs)
Paper, 101–103
Paracetamol, 511, 529
Paraoxonase 1 (PON1), 443, 444
Parkinson's disease, 179
Particle size, 170, 171
Particulate matter (PM), 165–197, 511, 512, 518, 524–526
Pathway, 290, 292, 299, 302, 303
PCA. *See* Principal component analysis (PCA)
PCBs. *See* Polychlorinated biphenyls (PCBs)
PcG/TrxG responsive elements (PREs/TREs), 503
PDGF. *See* Platelet-derived growth factor (PDGF)
Pediatric health, 147
Perfluorinated compounds, 488
Peroxisome proliferators, 511, 530
Persistent organochlorine compounds, 92
Personalized medical treatment strategies, 473, 485
Personalized medicine, 472, 486–487, 489
Personal protective equipment (PPE), 13, 15
Phagocytosis, 184, 187, 190
Pharmacogenetics, 438
Pharmacokinetics, 48, 51–54
Phase I
  cytochrome P-450 (CYP), 441
  flavin-dependent monooxygenases, 441
Phase II
  glutathione-S-transferase, 441, 442
  N-acetyltransferases, 441, 442
Phenotype phenylketonuria (PKU), 438
4-Phenylbutyric acid, 531
Phospholipid patterns, 482, 483
Phosphorylation, 502, 522
Phthalate monoesters, 93
Phthalates, 325–328, 347
Physiologically based toxicokinetic (PBTK) models, 489
PIWI-interacting RNAs (piRNAs), 500, 503–506, 510
Placental barrier, 322
Plasticized PVC, 88, 99, 103
Platelet-derived growth factor (PDGF), 319
$PM_{0.1}$, 170, 193, 195
$PM_{2.5}$, 170, 171, 173–176, 184, 188–193, 195, 196
PM_{10}, 167, 170–176, 178, 183, 184, 187–193, 196
p38 MAPK, 120
Poisons, 2, 15–17
Pollutants, 22–34, 36, 37, 41, 42
Polyaromatic hydrocarbons, 415
Polybrominated diphenyl ethers, 489
Polychlorinated biphenyls (PCBs), 253, 255, 258, 416, 417, 424–427
Polycomb group proteins (PcGs), 503
Polycomb repressive complex 2 (PRC2), 520

Polycyclic aromatic hydrocarbons (PAHs), 107–122, 414, 427–430, 533
Polymorphism, 172, 173
  dbSNP, 438
  haplotype, 438, 439
  non-synonymous, 439, 443
Polymorphonuclear neutrophils (PMN), 175, 181, 182
Polyvinyl chloride (PVC), 325, 326
PON1. *See* Paraoxonase 1 (PON1)
Posttranscriptional gene silencing (PTGS), 504–507
Potency, 292, 299–301
PPE. *See* Personal protective equipment (PPE)
Precautionary treatment, 472, 484
Prediction, 296–301
Principal component analysis (PCA), 480, 481
Programmed cell death, 116
Progressive degenerative disease, 518
Proliferation-activated receptor (PPAR), 530
Prostate, 317, 319, 329, 333, 342, 345, 348–350
  cancer, 323, 336, 348, 350
  development, 348–350
Protein-protein network, 447
Proteins, 135, 137–139, 142, 143, 145, 148–151, 438, 439, 441, 445, 447, 448
Proteomic biomarkers, 460, 462–463
Proto-oncogenes, 112, 113, 120, 142
Pseudoagouti mouse, 508
PTGS. *See* Posttranscriptional gene silencing (PTGS)
Public health, 134, 135, 151, 153
PVC. *See* Polyvinyl chloride (PVC)
Pyrophoric substances, 3

Q
Quantitative chemical health risk assessment, 382, 399, 403
Quantitative structure *in vitro-in vivo* relationship (QSIIR), 398–402
Quantitative structure-toxicity relationship (QSTR) model
  aquatic toxicity, 394–398
  commercial tools, 402–404
  database, 389–393
  statistical parameters, 395
  target properties, 384–385, 393, 398
  validation of, 385–387
  in vitro-in vivo relationship, 398–402
  workflow, 387–388
Quantum confinement, 224, 228
Quantum dots, 225, 229
Quantum effects, 224

R
Radiation, 337
Radioimmunoassay (RIA), 472, 473
Reactive oxygen species (ROS), 109, 113–115, 117, 121, 171, 180–184, 189–191, 195, 196, 509, 518, 524, 525
Recycling process, 101
Redox cycling, 115
Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH), 323
Repeated dose toxicity, 4
Reporter gene expression, 478
Reproductive toxicants, 518–524
Reproductive toxicity, 4
Residual oil fly ash (ROFA), 168, 169, 175, 177, 185, 187–190, 193
Restriction landmark genomic sequencing (RLGS), 520, 522
Risk assessment, 6–9, 11, 13–14, 19, 362, 367–374, 459–467
Risk control, 11, 14
Risk management for hazardous chemicals, 5–8, 19
RLGS. *See* Restriction landmark genomic sequencing (RLGS)
RNA
  expression, 450
RNA-induced silencing complex (RISC), 504
RNA interference (RNAi), 504, 506
Rodent model, 347, 349
ROFA. *See* Residual oil fly ash (ROFA)
ROS. *See* Reactive oxygen species (ROS)
Rule-based systems, 382

S
S-Adenosyl-methionine (SAM), 508, 511, 512, 514
Safety data sheets (SDS), 3, 5, 7, 12, 15
Saxitoxin, 417, 430
Schulz, Hugo
  father of hormesis, 553
Selenium (Se), 134, 151
Self-heating substances, 3
Self-reactive substances, 3
Seminiferous tubules, 317, 325, 326, 328, 330–337, 340, 346, 350
Senescence, 317, 340
Sensitisers, 4
Sensitization, 289–304
Sertoli cells, 318, 319, 325–327, 329–333, 335, 336, 340, 342, 347, 350
Shotgun lipidomics, 482, 484, 485
Signaling pathways, 139

Silver (Ag), 135
Single dose toxicity, 4
Skin
  penetration, 238
  sensitization, 289, 291, 293–301, 303
Small fish model, 35, 38–39, 42, 43
Small interfering RNAs (siRNAs), 500, 503, 504, 506, 510
Small noncoding RNAs (ncRNAs), 500, 503–507, 510
Social environment, 450
Soft corona, 233
Solid, 3
Source, 166–168, 181, 195
Southam, Chester
  named hormesis, 552
Speciation, 134, 152
Species sensitivity distribution, 26–29
Sperm, 317, 326, 328, 331–333, 335–340, 343, 344, 350
Spermatogenesis, 319, 320, 330–332, 335, 337, 350
Sperm biomarkers, 337–339
Spontaneous abortions, 148
SRY gene, 318
Staple foods, 98, 103
Stern layer, 228
Stress genes, 138, 148
Stroke, 174
Strontium (Sr), 135
Sulfotransferases (SULT), 115
Sumoylation, 502
Sunscreens, 229, 238
Superoxide ($\bullet O_2^-$), 176, 181, 195, 196
Superoxide anion radical ($O_2^-\bullet$), 113–115, 121
Superoxide dismutase, 113
Surface area, 171, 178, 180, 197
Surface plasmone resonance (SPR), 225, 228
Surface plasmonics, 228
Susceptibility, 137, 139, 152, 171–172, 193
Systemic inflammation, 173, 175, 178, 180

T
Tamoxifen, 511, 529–530
Targeted metabolomics, 477–479
Target organ systemic toxicity (TOST), 4
Target properties, 384–385, 393, 398
T cells, 294, 297–299, 303
TCR. *See* Transcription-coupled repair (TCR)
Tellurium (Te), 135
Teratogenic effects, 142
Testicular descent, 317, 319–321, 340, 350
Testicular dysgenesis syndrome, 320, 324–325
Testicular germ cell tumor (TGCT), 345–348, 350
Testis, 317–321, 324, 325, 327–332, 335, 336, 338–340, 343–348, 350
Testosterone, 318, 319, 322, 324, 326, 328, 329, 331, 332, 334–337, 339, 340, 348, 349
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), 512, 533
Tetrahymena pyriformis, 394, 396
Tetrodotox, 417, 430
TGF. *See* Transforming growth factor (TGF)
T-G mismatch glycosylase, 502
TGS. *See* Transcriptional gene silencing (TGS)
Thalidomide, 417, 430
Thallium (Tl), 135
Tiered testing approach, 237
Tight junctions (TJ), 329
Tin (Sn), 135
Titanium (Ti), 135
Titanium dioxide, 168, 169, 182, 196
T lymphocyte/T cell, 254, 258, 259, 261, 268–271, 274
Tobacco-associated lung tumors, 113
Tobacco carcinogens, 527
Tolerable daily intake (TDI), 89, 94
Toluene diisocyanate (TDI), 253, 265, 266
Toxaphene, 416–418, 420
Toxicant-specific profiles, 510
Toxicities of chiral pollutants, 414, 418–431
Toxicity, 133–153, 381–405
Toxic metals, 133–153
Toxicodynamics and pharmacodynamics, 439, 444–445
Toxicokinetics, 440
Toxics, 2–4, 10, 18
Training, competency, 7, 11–13, 19
Transcriptional gene silencing (TGS), 506, 507
Transcription-coupled repair (TCR), 116, 118
Transforming growth factor- (TGF-), 172, 193
Transgenerational effects, 316, 317, 343–345, 350, 521, 523
Transgenerational epigenetic effect, 523
Transition metals, 168, 169, 171, 174, 181, 191, 192
Translesional synthesis, 120
Trichostatin A, 517, 522
Trithorax group of proteins (TrxGs), 503
Troponin, 473
Tumor induction, 54–55, 64
Tumor suppressor genes, 112, 113, 122
Tumor necrosis factor- (TNF), 121, 177, 179, 182, 183, 187–196

U
Ubiquitination, 502, 514
Ultra performance liquid chromatography (UPLC), 477, 482
Uncertainty factor (UF), 277
Untargeted metabolomics, 477–479
Uranium (U), 135
UV filters, 229

V
Validation, 384–388, 391, 393–397, 399, 400, 403–405, 460, 465–467
Valproic acid (VPA), 511, 512, 528
Vanadium (V), 135
Vascular cell adhesion molecule-1 (VCAM-1), 175, 176, 195
Vegetables, 98
Vinclozolin, 329, 341, 344–346, 522–524
Vinyl carbamate, 527
VITOSENS®, 296, 297, 299
von Willebrand factor (vWF), 175

W
Waste management, 5, 17
Water-activated flammable gases, 3
Water quality, 23
  guidelines, 34
  objective, 32, 34
Water quality criteria (WQC), 22–34
Weight of evidence (WOE), 271, 273, 276, 277, 279
Wolffian duct, 318, 319
World Health Organization, 143
Wy-14,643, 530

X
X-chromosome inactivation, 501
Xenoestrogen, 519
Xenotransplant model, 328

Y
Y-polymerases, 112

Z
Zinc (Zn), 134, 152