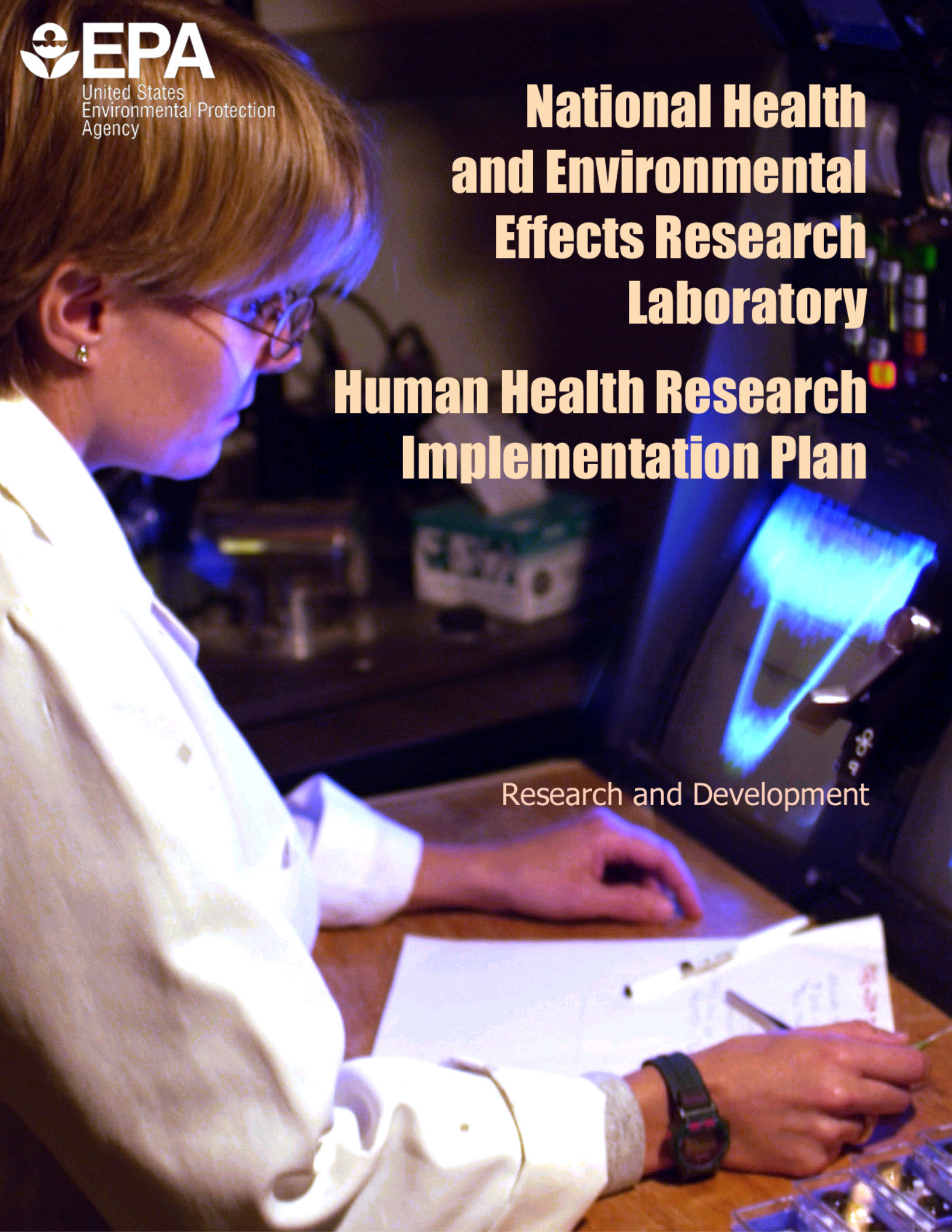

United States Environmental Protection Agency

National Health and Environmental Effects Research Laboratory

Human Health Research Implementation Plan

Research and Development

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National Health and Environmental Effects Research Laboratory

Human Health Research Implementation Plan

US Environmental Protection Agency
National Health and Environmental Effects Research Laboratory
Office of Research and Development
Research Triangle Park, NC 27711

Foreword

The National Health and Environmental Effects Research Laboratory (NHEERL), as part of the Environmental Protection Agency's Office of Research and Development (ORD), is responsible for conducting research to improve the risk assessment of chemicals for potential effects on human health. This research is intended to address key Agency problems in a timely and responsive manner. To meet this responsibility, NHEERL is developing research implementation plans to achieve the following objectives:

- Optimizing responsiveness of research activities to Agency needs,
- Sharpening the focus of research programs where needed,
- Providing a forum for engagement of scientific staff on issues and approaches,
- Focusing on multi-year planning explicitly linked to Agency performance goals, and
- Providing a mechanism for prioritizing research.

This approach builds on the ORD planning process that identifies and prioritizes research needs. Current areas for research include protection of susceptible subpopulations, harmonization of risk assessment approaches, and improving aggregate and cumulative risk assessment in support of the Agency's Program and Regional Offices and legislative mandates, including the Safe Water Drinking Water Act, Clear Air Act, Toxic Substances Control Act, and Federal Insecticide Fungicide and Rodenticide Act.

This document identifies the scientific problems and research that will be conducted concerning human health. The ultimate goal of this research is to develop scientifically valid approaches for improving human health risk assessment. This document was developed by representatives from NHEERL research divisions and peer-reviewed by scientists from other ORD laboratories and centers and EPA Regional and Program Offices. This document was also reviewed by scientists external to the Agency. This implementation plan is intended to reflect research that will be conducted over the next several years. As progress is made in achieving the goals outlined in this document, it will be updated to address new and remaining human health challenges.

Lawrence W. Reiter
Director
National Health and Environmental Effects Research Laboratory

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Acknowledgments

Preparation of the *NHEERL Human Health Implementation Plan* was coordinated by the NHEERL Human Health Research Executive Committee whose members are listed on the next page. Research themes for the proposals described in this document were developed at a scientist-to-scientist meeting held at Research Triangle Park in November 2001. Participants at that meeting included scientists from NHEERL, as well as scientists from other ORD laboratories and Centers which include:

- National Center for Environmental Assessment (NCEA),
- National Center for Environmental Research (NCER),
- National Exposure Research Laboratory (NERL), and the
- National Risk Management Research Laboratory (NRMRL).

Scientists from several Regional Offices, the Office of Air and Radiation, Office of Water, and the Office of Prevention, Pesticides, and Toxic Substances were also in attendance. Based on recommendations generated from the scientist-to-scientist meeting, research proposals were generated and reviewed by the NHEERL Human Health Research Executive Committee, as well as the following non-NHEERL scientists:

- Rick Hertzberg (Region 4),
- Kim Hoang (Region 9),
- Marc Rigas (NERL), Chris Saint (NCER),
- Jennifer Seed (Office of Toxic Substances),
- Sophia Serda (Region 9),
- Linda Sheldon (NERL),
- Michel Stevens (NCEA),
- Cynthia Sonich-Mullin (NCEA), and
- Edward Washburn (Office of Science Policy).

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Acronyms

ADHD	Attention Deficit Hyperactivity Disorder
AMD	Age-Related Macular Degeneration
APG	Annual Performance Goal
APM	Annual Performance Measure
As	Arsenic
BBDR	Biologically Based Dose Response
CCL	Contaminant Chemical List
CDC	Centers for Disease Control and Prevention
ChE	Cholinesterase or acetylcholinesterase
CNS	Central Nervous System
COPD	Chronic Obstructive Pulmonary Disease
CVD	Cardiovascular Disease
CYP	Cytochrome P-450
2,4-D	2,4-Dichlorophenoxy Acid
DEHP	Diesel Exhaust Particles
DBP	Disinfection By-Product
EBIFs	Ergosterol Biosynthesis Inhibiting Fungicides
EDC	Endocrine Disrupting Chemical
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FQPA	Food Quality Protection Act
FSH	Follicle Stimulating Hormone
GJC	Gap Junction Communication
γ-GCS	γ-Glutamyl Cysteine Synthetase/Ligase
GnRH	Gonadotrophin-Releasing Hormone
GPRA	Government Performance and Results Act
GST-O	Glutathione-S-Transferase Omega
HAAs	Haloacetic Acids
HAPs	Hazardous Air Pollutants
HO-1	Heme Oxygenase-1
iAs	Inorganic Arsenic
IgE	Immunoglobulin E
ICC	Interagency Coordinating Committee
IUGR	Intrauterine Growth Retardation
LH	Luteinizing Hormone
LTGs	Long-Term Goal
MAPK	Mitogen-activated protein kinase
MOA	Mode of Action
MYPs	Multi-Year Plans
NCEA	National Center for Environmental Assessment
NCER	National Center for Environmental Research
NCS	National Children's Study
NE	Noradrenergic
NERL	National Exposure Research Laboratory
NHEERL	National Health and Environmental Effects Research Laboratory
NIH	National Institutes of Health
NTFs	Neurotrophic Factors

NOAEL	No-Observed-Adverse-Effect-Level
NOEL	No-Observed-Effect-Level
NQO-1	NADPH Quinone Oxidoreductase-1
NRC	National Research Council
NRF2	Nuclear Factor Erythroid-Derived 2, Like 2
NRMRL	National Risk Management Research Laboratory
OCHP	Office of Children's Health Protection
OP	Organophosphate
OPP	Office of Pesticide Programs
OPPTS	Office of Prevention, Pesticides, and Toxic Substances
ORD	Office of Research and Development
OW	Office of Water
PBPK models	Physiologically Based, Pharmacokinetic Models
PCBs	Polychlorinated Biphenyls
PCR	Polymerase Chain Reaction
PD models	Pharmacodynamic Models
PK models	Pharmacokinetic models
PKC	Protein Kinase C
PM	Particulate Matter
POD	Point of Departure
RfD	Reference Dose
RPE	Retinal Pigmented Epithelium
ROS	Reactive Oxygen Species
SAR	Structure Activity Relationship
SH Rats	Spontaneous Hypertensive Rats
STAR	Science to Achieve Results
TSH	Thyroid Stimulating Hormone
TSCA	Toxic Substances Control Act
US	United States
WKY Rats	Normotensive Wistar-Kyoto Rats
XMEs	Xenobiotic Metabolizing Enzymes

Executive Summary

This document describes the implementation plan for human health research within the National Health and Environmental Effects Research Laboratory (NHEERL) for the next 8-10 years beginning in FY02. Human health research in the Office of Research and Development (ORD) of the Environmental Protection Agency (the Agency) is based on needs and goals established by the Agency in response to the Government Performance and Results Act (GPRA). The human health research described in this document aims to provide broad, fundamental scientific information that will improve understanding of problem-driven issues arising from risk assessment in the Agency's Program and Regional Offices. Human health research supports the identification of fundamental mechanisms of toxicity and development of methods and models to elucidate key scientific uncertainties important to understanding and predicting the effects of environmental agents on human health.

This implementation plan on human health research provides a detailed approach that NHEERL will take to address scientific uncertainties described in the *ORD's Human Health Research Strategy* document, which includes harmonizing the use of mechanistic data in risk assessment, protecting the health of susceptible subpopulations, and determining risk of exposure to multiple chemicals. This document also outlines in detail how NHEERL's human health research will meet the Annual Performance Goals (APGs) and Annual Performance Measures (APMs) described in ORD's *Human Health Research Multi-Year Plan.* NHEERL's human health program is organized around program projects, which are multi-investigator, multi-disciplinary efforts designed to address major research themes. Programs projects were developed by teams and subject to internal and external review.

Research on Harmonization of Cancer and Non-Cancer Risk Assessments

The assessment of health risk from exposures to environmental agents has traditionally depended on whether the response is a cancer or non-cancer health effect. However, there is a growing consensus that there is a need to develop a consistent, flexible set of principles for using and drawing inferences from scientific information in risk assessment. NHEERL's research in this area will focus on understanding the biological events that precede toxic or adverse effects and identifying common or similar modes of action across cancer and non-cancer endpoints that could provide the basis for a harmonized approach for risk assessment. Mechanistic information is also crucial for reducing or replacing uncertainty factors in risk assessment, especially for interspecies extrapolation and for linking dosimetry models such as pharmacokinetic (PK) models with empirical or pharmacodynamic (PD) models for effects of chemicals having similar or differing modes or mechanisms of action. Three program projects were identified: (1) research on cell signaling pathways, (2) neuroendocrine mechanisms, and (3) P-450 and xenobiotic metabolizing enzymes.

Susceptible Subpopulations

The variability in responsiveness of humans to environmental pollutants can be associated with differences in biological susceptibility arising from intrinsic factors such as life stage and genetics or with acquired factors such as disease. Approaches to better identify and

characterize susceptible subpopulations by describing the biological basis underlying differential responsiveness is an overarching goal of NHEERL. Research on susceptible subpopulations will focus on developing a scientific understanding of the biological basis for differing responsiveness of subpopulations within the general population. NHEERL research on susceptible subpopulations will also address the role of life stage, genetic background, and preexisting disease on responsiveness to environmental agents. Seven program projects were identified: four focusing on life stage issues, one on asthma as a disease, and two on genetic components of susceptibility.

Cumulative Risk

Cumulative risk assessment is a major concern to many Agency Program and Regional Offices. NHEERL's research on cumulative risk will focus on providing data that may be used in the development and refinement of approaches to predict interactions of chemicals at low dose concentrations. Research in this area will utilize empirical and mechanistic data and models to develop strategies to predict the effects of chemical mixtures. The program project identified in this area will explore the limits of the additivity assumption that is the standard assumption in non-cancer risk assessment guidelines in the Agency. This research will also address mixtures of chemicals with common or dissimilar modes of action and will evaluate the effects of these mixtures in repeated exposure situations.

The research described in this *NHEERL Human Health Research Implementation Plan* not only involves interdisciplinary teams within NHEERL, but in many cases involves collaborations with scientists from other ORD Laboratories and Centers or Agency Program Offices. Each of the following sections attempts to link research in that program project to other Agency problem-driven research areas (i.e., air toxics, drinking water, pesticides, and toxic substances), as well as providing a narrative on the programmatic impact of the work and describing collaborative efforts with other Laboratories and Centers within ORD.

Section 1
Introduction

Purpose and Scope

This document, the *NHEERL Human Health Research Implementation Plan*, describes the framework and implementation plan for human health research within the National Health and Environmental Effects Research Laboratory (NHEERL) beginning in FY02. Human health research in the Office of Research and Development (ORD) of the Environmental Protection Agency (the Agency) is based on needs and goals established by the Agency in response to the Government Performance and Results Act (GPRA). The human health research described in this document is core research that aims to provide broad, fundamental scientific information that will improve understanding of problem-driven human health issues arising from risk assessment in the Agency's Program and Regional Offices. Core research focuses on developing and applying the best available science for addressing current and future environmental hazards, as well as on developing new approaches for protecting human health. NHEERL's human health research program supports the identification of fundamental mechanisms and development of methods and models to elucidate key scientific uncertainties important to understanding and predicting the effects of environmental agents on human health.

This implementation plan on human health describes the approach that NHEERL will take over the next 8-10 years to address the scientific uncertainties outlined in ORD*'s Human Health Research Strategy* document, which includes harmonized use of mechanistic data in risk assessment, protecting the health of susceptible subpopulations, and determining risk of exposure to multiple chemicals. This document also outlines in detail how NHEERL will meet the Annual Performance Goals (APGs) and Annual Performance Measures (APMs) described in ORD's *Human Health Research Multi-Year Plan*. Multi-Year Plans (MYPs) provide a framework to integrate research across ORD's Laboratories and Centers, a basis for creating annual plans, and a context within which to understand how decision-making in annual planning influences the ability of ORD to meet future goals and outcomes. MYPs identify Long-Term Goals (LTGs) that are to be addressed over an 8-10 year period while APGs and APMs represent milestones that must be accomplished in order to meet the LTGs.

Process for Developing this Implementation Plan

This implementation plan was developed through an iterative and interactive process leading to the development of program projects, which are multi-investigator, multi-disciplinary efforts designed to address the three major themes outlined in the ORD's *Human Health Research Strategy.* A Steering Committee oversaw the development of the program projects and was composed of two representatives from each health division (one manager, one scientist) and representatives from other ORD Laboratories/Centers and Regional Offices. NHEERL representatives on the Steering Committee served as the Executive Committee. ORD's *Human Health Research Strategy* and the FY02 *Human Health Research Multi-Year Plan* provided background information and guided strategic thinking as the planning effort progressed.

A scientist-to-scientist meeting was held in November, 2001, to fashion a limited number of integrated program projects responsive to the programmatic needs of the Agency. This meeting was attended by approximately eighty NHEERL scientists and twenty scientists from other parts of the Agency (i.e., ORD, Program and Regional Offices). Following this meeting, sixteen preproposals were submitted by research teams in NHEERL and reviewed by the Executive Committee. At least one review per preproposal was provided by a non-NHEERL scientist. The Executive Committee then recommended that eleven of the research teams develop full proposals; these evolved into the program projects described in this document.

The *NHEERL Human Health Research Implementation Plan* represents a work-in-progress which puts forward current thinking on major issues and ways to address them. As the research progresses, it will become increasingly apparent which ideas and approaches are leading successfully toward their goals and which may not be. The Executive Committee will convene a meeting of the researchers involved in this effort approximately every two years to present results and discuss future directions. It is expected that program projects will evolve over time in response to the success of the research effort and identification of new directions of significance to the Agency. Changes in work covered by the program projects at the Laboratory level will be linked to strategic revisions of the *ORD Human Health Research Multi-Year Plan.*

Section 2
Research Approach

Context for Research

The Agency is charged with the responsibility of protecting public health and the environment. To fulfill this mandate, the Agency uses the process of human health risk assessment to identify and characterize environmentally related human health problems. Human health risk assessment provides a qualitative and quantitative characterization of the relationship between environmental exposures and effects observed in exposed individuals. In 1983, the National Research Council (NRC) described four primary steps in the process of risk assessment: hazard identification, dose-response assessment, exposure assessment, and risk characterization. Risk assessment is the primary scientific input to the risk management process, which involves the recognition of a potential new risk and development, selection and implementation of Agency actions to address the risk. Risk management often considers a wide variety of other factors. The overall process of risk assessment and risk management is often called the risk assessment/risk management paradigm.

Risk assessment has become an integral component of environmental decision-making under the statutes implemented by the Agency. There are many uncertainties associated with the risk assessment process because of severe limitations in available data and the complex interactions between the sources and environmental concentrations of contaminants, dose at the target site, and response. These uncertainties frequently result in the use of default assumptions and uncertainty factors in risk assessments. NHEERL's human health research program is based on the assumption that major uncertainties in risk assessment can be reduced by understanding the fundamental determinants of dose and the basic biological changes that follow exposure to a chemical.

Human Health Research Themes

- ❑ Use of Mechanistic Data in Risk Assessment
- ❑ Protect Susceptible Subpopulations
- ❑ Assess Cumulative Risk

Based on input from Regional and Program Office risk assessors, human health research at NHEERL will focus on three major themes as described in the ORD's *Human Health Research Strategy* and the *Human Health Research Multi-Year Plan:* (1) the use of mechanistic data in risk assessments, (2) protection of susceptible subpopulations, and (3) assessment of cumulative risk. Research on harmonizing risk assessment approaches will lead to a common set of principles and guidelines for drawing inferences about risk from available scientific information. The overall goal of this research, which is described in Section 3 of this document, is that Program and Regional Office risk assessors will use mechanistic data in a harmonized manner for risk assessments for all health endpoints. NHEERL will also focus on developing a scientific understanding of the biological basis for differing responsiveness of subpopulations within the general population. Research on biological susceptibility, which is described in Section 4, will focus on the role of intrinsic factors such as life stage and genetic background and extrinsic factors such as preexisting disease on responsiveness to environmental pollutants. NHEERL's human health

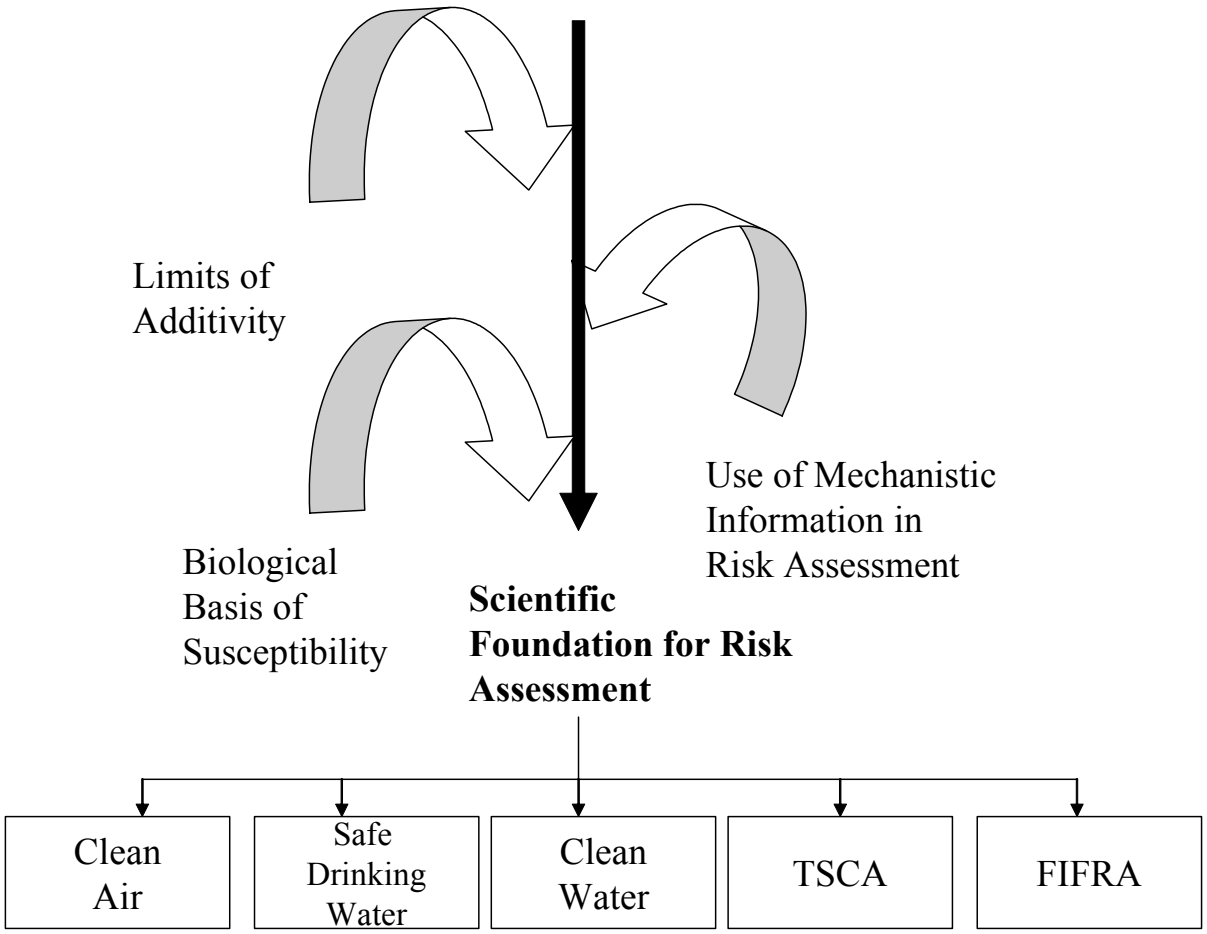

Figure 1 Mandates for Human Health Research

research program will also address the fact that humans are exposed to mixtures of pollutants from multiple sources. This research will provide scientific support for decisions concerning exposure to a multiple pollutants by assessing additivity as the default assumption describing interactions between chemicals in mixtures (see Section 5 of this document).

Authorization and Mandate for Human Health Research

One of the reasons for focusing on human health research is that virtually all of the Agency's major legislative mandates (those which require the Agency to promulgate regulations to protect the public health and welfare from environmental contaminants) require that the Agency develop human health risk assessments. These laws (and amendments) include the Clean Air Act; the Safe Drinking Water Act; the Clean Water Act; the Toxics Substances Control Act (TSCA); the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA); the Resources Conservation and Recovery Act; the Comprehensive Environmental Response, Compensation, and Liability Act; the Superfund Amendments Reauthorization Act; and the Food Quality Protection Act (FQPA). In addition, Congress enacted legislation in 1988 which mandated that the Agency undertake Research to Improve Health Risk Assessment. NHEERL's research to harmonize the use of mechanistic data in risk assessment, protect susceptible subpopulations, and assess cumulative risk will contribute to building the scientific framework for human health

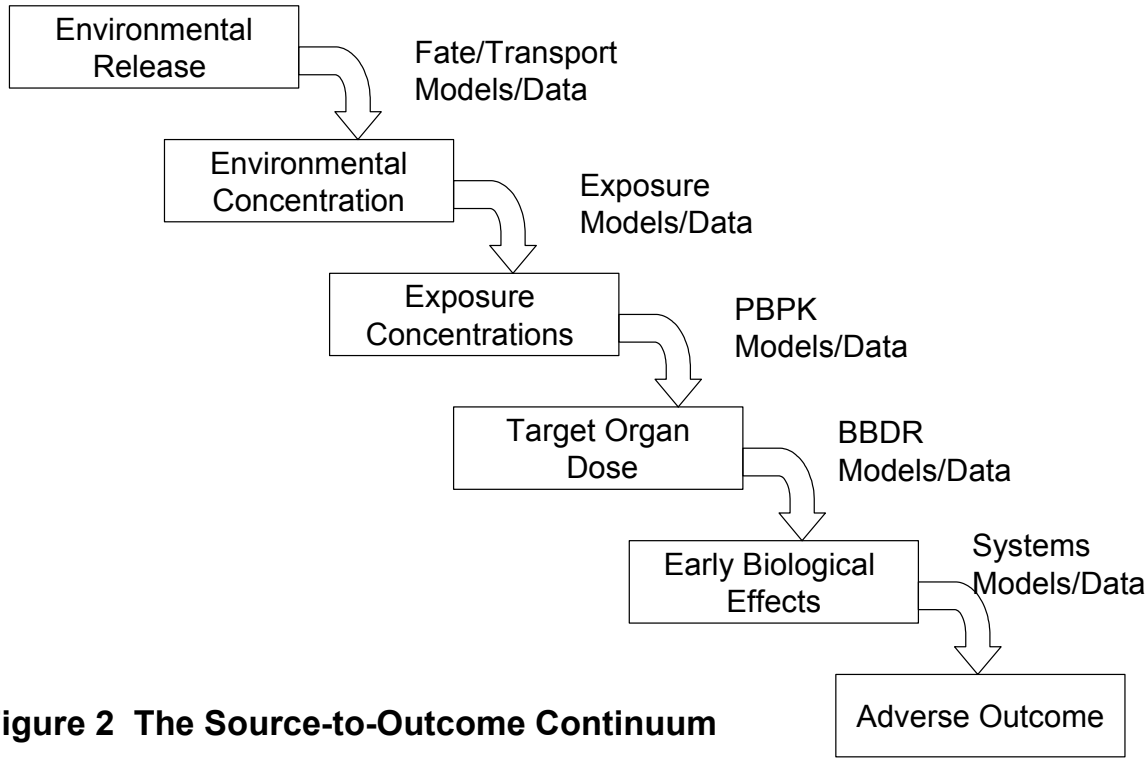

Figure 2 The Source-to-Outcome Continuum

risk assessments required by various legislative mandates (Figure 1). Of particular relevance to the human health research program is the that the FQPA requires children's risks to pesticide exposures be considered during the tolerance setting process. FQPA requires the development of scientific information necessary to "...ensure that there is a reasonable certainty that no harm will result to infants and children from aggregate exposure to the pesticide chemical residue...." Thus, information on children's exposure is required to (1) consider the susceptibility of children to increased exposure and (2) account for aggregate exposures to pesticides from all sources, including food, drinking water, and applications of pesticides in homes, schools, daycare centers, and other micro-environments. Because of these FQPA mandates, the results of NHEERL's research on susceptible subpopulations will be important for risk assessments by the Office of Prevention, Pesticides, and Toxic Substances (OPPTS). This Office is required to establish allowable levels of exposure to pesticides and toxic chemicals and protect the most susceptible subpopulations. The Safe Drinking Water Act Amendments (1996) also requires consideration of susceptible subpopulations, including children, when setting standards for chemicals and other contaminants in drinking water. Section 408 of the Federal Food, Drug, and Cosmetic Act, as amended by FQPA, identified several research issues related to the determination of risk of exposures to pesticides, including aggregate exposure and cumulative risk, susceptible subpopulations, an additional factor of 10 margin of safety for children, and questions related to extrapolation of data in risk assessment.

Multidisciplinary Human Health Research at ORD

Research on human health at NHEERL is viewed as part of a multidisciplinary research program within ORD that addresses linkages lying along a continuum from the

source of an agent through exposure and dose to adverse effects or outcomes (Figure 2). Research at NHEERL focuses on developing physiologically based, pharmacokinetic (PBPK) models to determine target organ dose, biologically based dose response (BBDR) models to determine early biological effects in pathways associated with toxic effects, and models to predict adverse effects. NHEERL human health research aims to reduce uncertainties in the risk assessment process, including extrapolation across species, extrapolation from short- to long-term lifetime exposures, and variability of response within the human population. For purposes of dose assessment, NHEERL works with the National Exposure Research Laboratory (NERL) to develop pharmacokinetic (PK) models to estimate internal dose metrics and establish the basis for metabolic differences between species. The National Center for Environmental Assessment (NCEA) performs complex risk assessments of national interest and develops risk assessment methods, databases, and tools based on results from NHEERL and other laboratories. The National Risk Management Research Laboratory (NRMRL) focuses on providing the most effective and useful risk management options and increasing linkage between risk assessment and risk management efforts. Intramural research conducted by NHEERL is complemented by extramural research sponsored by the Agency's National Center for Environmental Research (NCER). Through the Science to Achieve Results (STAR) Program, NCER supports grants that focus on specific research needs consistent with the mission of the Agency. Specific linkages between NHEERL research on human health and other Laboratories/Centers within ORD, as well as with other research groups, is described in greater detail in the following sections. Resource allocation for each program project based on FY03 FTE projections can be found in Appendix A.

Section 3
Harmonization of Cancer and Non-Cancer Risk Assessments

3.1 Problem

The assessment of health risks from exposures to environmental agents has traditionally depended on whether the response is a cancer or non-cancer health effect. However, the 1997 report *Risk Management in Regulatory Decision Making* from the Commission on Risk Assessment and Risk Management concluded that the simple dichotomy between cancer and non-cancer risk assessment is not fully supportable by current scientific evidence because it results in expressions of risk that are not directly comparable and differ significantly in defining maximal exposures considered to have negligible risk. In addition, the National Research Council's report on *Science and Judgment in Risk Assessment* (NRC, 1994) noted the importance of a risk assessment approach that is less fragmented, more consistent in application of similar concepts, and more holistic than endpoint-specific guidelines. The Agency's Risk Assessment Forum has also been actively involved in reexamining approaches to cancer and non-cancer risk assessment by sponsoring internal colloquia to encourage input and discussion between scientists and risk assessors. One conclusion from these discussions is that there is a need to develop a consistent, flexible set of principles and guidelines for using and drawing inferences from scientific information in risk assessment.

One issue related to the disparity in approaches to cancer and non-cancer risk assessments is the limited understanding of the mode (MOA) or mechanism of action of compounds. This lack of knowledge has largely been due to limitations in the experimental designs, methods, and animal models that have been used to support hazard identification and dose-response phases of human health risk assessments. Therefore, understanding an agent's MOA is key to more accurate prediction and characterization of hazard and risk and is the basis for developing harmonized risk assessment approaches for all health endpoints. At present, risk assessment guidelines for cancer and non-cancer endpoints differ with respect to the use of MOA information. Guidelines for non-cancer effects such as reproductive toxicity (US EPA, 1996) and neurotoxicity (US EPA, 1998) use mechanistic information largely in a weight-of-evidence framework to strengthen the evidence that a human hazard could exist. For example, mechanistic data could be used to support findings from an epidemiological study or results from animal toxicity studies. Mechanistic data may also be used on a case-by-case basis to reduce the magnitude of uncertainty factors used to calculate the Reference Dose (RfD), Reference Concentration, or Benchmark Dose. For example, if the same MOA could be demonstrated to occur in humans and animals, the uncertainty factor for animal-to-human extrapolation could be reduced in the risk assessment.

The use of MOA information is spelled out in detail in the draft final Guidelines for Carcinogen Risk Assessment (US EPA, 2003). As in the case of non-cancer endpoints, MOA data can be used in a weight-of-evidence framework to support the evidence for carcinogenic hazard potential based on epidemiological or animal toxicity studies. On the other hand, these guidelines note that MOA information is key to addressing various default options in the risk assessment. BBDRs using

mechanistic information can be used to relate dose and response on an agent-specific basis and to extrapolate to lower dose levels, if needed. MOA data can be crucial for establishing the point of departure (POD) for risk assessment, the linearity or non-linearity of the dose response relationship, and the biological plausibility of a response. MOA data are also important for determining key events that are of increased concern to susceptible subpopulations, such as children, and extrapolation from animals to humans. MOA information plays an important role in developing PK models to estimate dose in target tissues and helps define relative sensitivity of various tissues to carcinogens. Unlike risk assessment guidelines for non-cancer endpoints, the draft Cancer Guidelines contain a framework for the analysis of carcinogenic MOA information, including steps necessary to define the postulated MOA, identification of key events, and assessment of experimental evidence supporting the postulated MOA.

There is a clear need to develop a common approach for the use of MOA data in risk assessment (Bogdnanffy et al., 2001). In the context of this implementation plan, harmonization refers to developing a consistent, flexible set of principles for using and drawing inferences from available information on MOA to support risk assessment. **"Mode of action"** is defined as a series of key events starting with interaction of an agent with a cell and proceeding through functional and anatomical changes to result in an adverse effect**. "Mechanism of action"** implies a more detailed understanding and description of the key events, including the molecular level. Mechanism of action often describes how a chemical works at all levels of biological organization. For the purposes of this document, mode and mechanism of action are used interchangeably in this document. **"Key events"** are defined as empirically derived precursor steps linking the proximal event to the adverse outcome. The sequence of key events leading to an adverse effect at a given internal dose is also known as the **"toxicity pathway."** Toxicities arise from a range of factors that may be specific to the chemical or chemical class. The physiology of the organism and other environmental factors may affect the expression of toxicity. A framework for harmonization of risk assessment approaches must facilitate incorporation of data that accounts for these factors.

3.2 Goals

The overarching goal of mechanistic research at NHEERL is to help derive a commonly accepted set of principles defining how MOA information can be used in risk assessment, particularly as it relates to extrapolation in risk assessment. The NHEERL human health research implementation plan addresses how MOA information can be used in a more consistent way to improve dose-response assessment for the following:

- For various types of cancer and non-cancer endpoints,
- For chemicals producing different toxicities by a similar MOA, and
- For chemicals producing similar toxicities by different MOAs.

This research also addresses how mechanistic data can be used in risk assessments to

- Compare adverse outcome across toxicities,
- Select the POD and approaches for low-dose extrapolation, and
- Account for inter- and intra-species differences in PK and PD capabilities for all health endpoints.

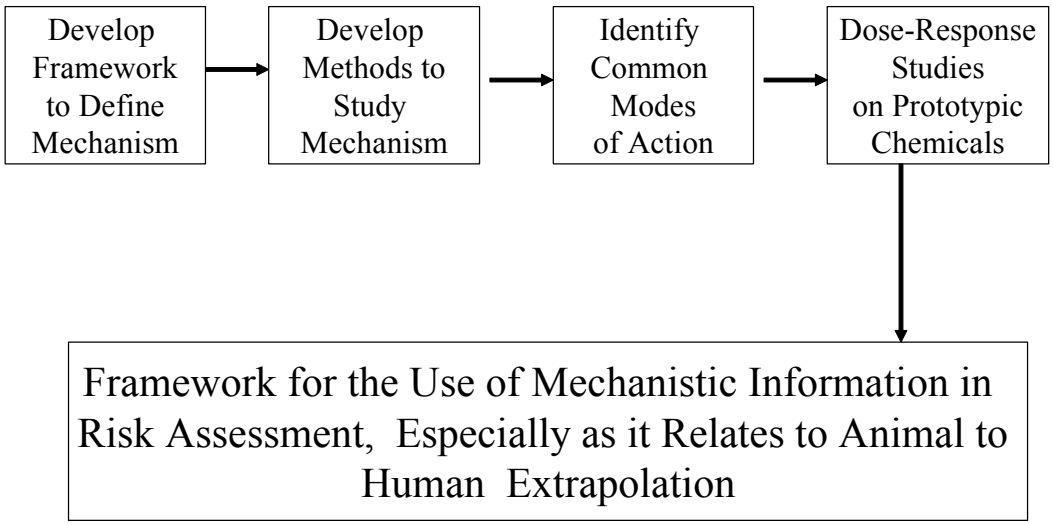

Figure 3 Critical Path for Harmonization Research

3.3 Critical Path

Consistent utilization of MOA information in risk assessment involves several critical steps, as outlined in Figure 3. In order to achieve a more harmonized approach for risk assessment based on the use of MOA data, there must first be a common framework for defining MOA. Questions to be addressed include the following:

- How much evidence is needed to show that a substance acts via a particular MOA in animals and its applicability to humans?
- How to show that two toxic manifestations caused by the same substance were produced by different MOAs?
- How to show that a common effect produced by two chemicals may have been caused by two different MOAs?
- What should be the dose-response assessment approach for a chemical that produces multiple toxic manifestations, but through a similar MOA?

It is also highly probable that a key element of research on the use of MOA data in risk assessments will be the application of emerging technologies, especially in molecular biology. Methods and approaches based on proteomics and genomics will likely prove crucial in testing hypotheses concerning common key events that lead to both cancer and non-cancer effects. Of particular concern will be approaches to quantify changes in gene and protein expression in toxicological studies and to interpret such changes in a risk assessment context. This will determine the extent to which these emerging technologies could be used to inform mechanistic questions in risk assessment. A research program applying molecular biological techniques with mathematical and computer models for prediction of effect and understanding MOA is being developed by ORD and is described in the draft document *A Framework for a Computational ToxicologyResearch Program in ORD.*

Once a framework has been developed to define MOA for use in risk assessment, work will be done to identify possible common modes of action for chemicals producing cancer and non-cancer effects.

Research will develop a clear understanding of the biological changes that occur following delivery of the active chemical moiety to target sites and the relationship of response to dose. Emphasis will be placed on identifying possible common key events (e.g., cell proliferation, receptor interaction, response to injury or stress) for prototypic chemicals that have both cancer and non-cancer effects. The overall objective of this research will be to demonstrate the feasibility of determining potential common precursor MOAs for cancer and non-cancer effects. This information will provide the basis for subsequent research to characterize precursor steps for several prototypic chemicals. Issues that will be addressed by this research include the following:

- How MOA data at the low end of the dose-response curve can inform decisions about the most appropriate risk assessment model,
- How MOA data can inform decisions about the presence or absence of a threshold, and
- How mechanistic information can be used to select the POD.

APGs and APMs related to research on the use of mechanistic data in risk assessment in the ORD *Human Health Research Multi-Year Plan* cross referenced to each program project can be found in Appendix B.

3.4 Program Projects

Research on harmonization of risk assessment consists of three program projects addressing MOAs and their use in risk assessment. Program project 1 addresses the hypothesis that exposure to different classes of chemicals will disrupt a common key event, i.e., mitogen-activated protein kinase (MAPK), in multiple tissues leading to a range of toxicities. Program project 2 addresses the hypothesis that disruption of luteinizing hormone (LH) secretion serves as a common MOA for altered fertility, reproductive disease, and cancer of the reproductive system in animals and humans. Program project 3 addresses the hypothesis that modulation of cytochrome P-450s and other xenobiotic metabolizing enzymes (XMEs) will lead to common MOAs for multiple toxicities. A fourth program project "Environmental and Genetic Interactions in Hypertensive Rats: Oxidative Stress as a Common Susceptibility Attribute for Non-Cancer Risks" contains some mechanistic research concerning oxidative stress as a common MOA. However, the focus of this program project is primarily on questions related to differential responsiveness to chemical exposure, i.e., susceptible subpopulations (see Section 4 for details). These four themes were selected because they involve toxicity pathways associated with prominent adverse health effects of high priority to risk assessors (i.e., cancer, reproductive and pulmonary toxicity, neurotoxicity).

Program Project 1: Harmonization of Cancer and Non-Cancer Risk Assessment: Disruption of Mitogen-Activated Protein (MAPK) Signaling as a Common MOA for Environmental Toxicants

Objectives:

- Use both *in vitro* and *in vivo* models to demonstrate that diverse groups of chemical compounds can disrupt MAPK signaling in multiple tissues,
- Examine the disruption of MAPK signaling as a common MOA leading to the expression of multiple adverse effects using pharmacologic and molecular manipulations of the MAPK signaling cascade as well as environmental toxicants, and
- Demonstrate that changes in MAPK signaling studied *in vitro* can be used to extrapolate the MOA of chemicals *in vivo*. Determine the degree of homology of response between animals and humans.

Scientific Approach

The transmission of external signals to the interior of the cell is a fundamental process in the regulation of cellular responses to physiological stimuli. Virtually every reaction that a cell makes in response to its environment is regulated by signaling processes. These signaling pathways exist in all cell types and determine the activity of the cell ranging from quiescence to fully stimulated. Cell signaling pathways are known to be upstream mediators of a number of basic biological processes including cell cycling, proliferation, migration, differentiation, plasticity, inflammation, and cell death. Signal transduction pathways are critical intermediates, accepting and integrating extracellular information with the nucleus to affect gene transcription and invoke a biological response. Thus, cell signaling pathways have the potential to serve as early and sensitive indicators of biological perturbation (NRC, 2000). In addition, understanding the signal transduction pathways that underlie the biological response to environmental and chemical insult is critical to identifying the MOA leading to adverse health effects.

Protein kinases are signaling molecules that interact with other messenger systems and form a highly structured network to integrate cell functions in an organism. These signaling pathways consist of proteins grouped in "cascade" fashion which typically originate in the cell surface and reach into the cell cytosol and nucleus. Each of the proteins has a structural and/or catalytic function that fits in the overall pathway to attenuate the specificity, magnitude, and duration of the signal being transmitted. The pathway to be studied in this proposal, the MAPK cascade, contains receptor kinases as well as serine/threonine, tyrosine, and dual specificity kinases with multiple regulatory domains that determine the specificity of the interactions (Chang and Karin, 2001).

A number of studies conducted by NHEERL scientists and others have demonstrated that environmental contaminants can disrupt signaling processes in target cell types in a variety of organ systems, including the lung, the nervous system, the reproductive organs, and the developing fetus. Although these compounds differ markedly in their physicochemical properties, they appear to share a common MOA in their ability to damage cell signaling by interfering with or enhancing signal initiation, transduction, or termination. Physiologically inappropriate

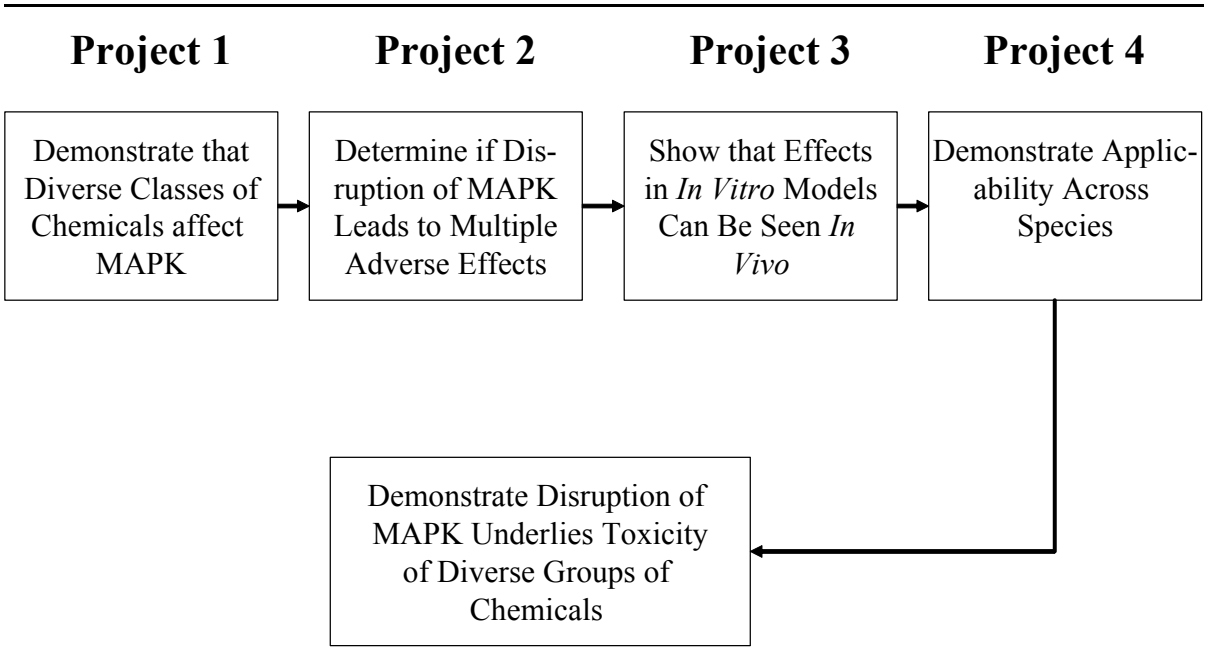

Figure 4 Critical Path for Research on Cell Signaling

signaling appears to result in a broad range of toxic responses, including apoptosis, inflammation, metaplasia, hyperplasia, hypertrophy, and neoplasia. Because MAPKs are involved in these basic cellular processes, it is proposed that disruption of MAPK signaling may be a common MOA that underlies the multiple adverse effects observed in diverse tissues. The critical path for research on cell signaling is illustrated in Figure 4.

Project 1 - Evaluation of MAPK Signaling after Exposure to Environmental Toxicants. External stimuli play a major role in regulating complex intracellular processes such as gene expression, cell proliferation, growth, differentiation, and death. These stimuli include cell-substrate adhesion, growth factors, hormones, neurotransmitters, and cytokines that stimulate a plethora of receptors. Environmental toxicants can act at many of these sites to perturb intracellular signaling, and, depending on exposure conditions, tissue, and life stage, can result in wide variety of adverse effects. In order to harmonize risk assessment approaches it will be necessary to determine whether the array of possible upstream targets of toxicants have a common downstream response. In a multitude of cell types, activation of a variety of receptors (e.g., G-protein coupled receptors, ion channels, receptor tyrosine kinases and cytokine receptors) trigger the MAPK cascades which serve as central downstream integrators to modulate the cellular response to external stimuli. Research in this project will test the hypothesis that exposure to different classes of chemicals will disrupt MAPK signaling in multiple tissue types. This work is intended to determine whether effects on diverse upstream targets will lead to changes in a common downstream endpoint, MAPK, and provides the basis for continued testing of MAPK signaling as a common MOA.

The studies outlined in this project will use several different *in vitro* and *in vivo* models to understand the potential for altered upstream signaling to lead to changes in MAPK in embryonic tissue (developmental toxicity), brain tissue (neurotoxicity), pulmonary tissue (airway disease), and skin epithelium (cancer). Because several different model systems will be used, the chemicals to be examined

will vary. For each model system, chemicals with known adverse effects will be chosen based on existing data. Research in project 1 will demonstrate that known environmental toxicants which act on diverse cellular targets in multiple tissues ultimately lead to alterations in MAPK signaling. Based on considerations of dose-response and toxicity, chemicals and models which show a change in MAPK signaling at relevant doses will be chosen for further study in project 2. Research in project 1 will include the following:

- Effects of arsenic (As) on cell signaling in primary human keratinocytes and bladder epithelial and endothelial cells,
- Effects of particulate matter (PM) on MAPK cascade components inhuman airway epithelial cells,
- Effects of toxicants on ligand-receptor interactions and subsequent MAPK signaling events in the developing embryonic palate,
- Effects of developmental toxicants that alter protein kinase and MAPK signaling in whole embryo cultures,
- Effects of toxicants that perturb electrical excitability, integrin-receptor interactions or calcium regulation of MAPK signaling events in neuronal tissue, and
- Use of flow cytometry and confocal microscopy to detect MAPK signaling.

Project 2 - Disruption of MAPK Signaling as a Common MOA Leading to Expression of Multiple Adverse Effects. The studies in project 1 are designed to demonstrate that toxicant-induced effects on a variety of upstream targets ultimately converge to perturb MAPK signaling. The critical question to be addressed next is whether the observed changes in MAPK are related to the expression of toxicity in multiple tissues. It is clear that MAPK signaling is an important mediator of critical cellular functions including embryogenesis, cell proliferation, cell differentiation, and cell death. Thus, alterations in MAPK signaling is expected to be part of the MOA that leads to changes in critical cellular functions which are ultimately expressed as the adverse effect in the different tissues and model systems described above. This work will attempt to tie the changes in MAPK signaling identified in project 1 to consequences downstream from this critical signaling event. Research covered in project 2 will include the following:

- Studies on MAPK-induced disruption of cell cycle progression leading to augmented cell proliferation and carcinogenesis by arsenicals,
- Effects of PM metals on MAPK activation and subsequent gene transcription,
- Studies on toxicants that interfere with ligand-receptor interactions to alter critical signaling events in the developing embryo,
- Effects of disruption of MAPK signaling and craniofacial development by altering differentiation of neural crest cells after exposure to haloacetic acids (HAAs) *in vivo* and *in vitro,*
- Research to determine if disruption of cell signaling is a MOA by which developmental neurotoxicants alter neuronal differentiation and synaptogenesis,
- Studies to determine if disruption of cell signaling is a MOA by which developmental neurotoxicants interfere with activity-dependent plasticity in intact neural circuitry, and
- Use flow cytometry and confocal microscopy detection of MAPK signaling to demonstrate that alteration of MAPK affects basic cellular processes, including proliferation and apoptosis**.**

Project 3 - Use of *In Vitro* Signaling Data for Extrapolating the MOA of Environmental Toxicants *In Vivo*. The Agency depends on mechanistic toxicological research to provide biological plausibility to strengthen the knowledge base used in risk assessments in a weight-of-evidence context. However, much of the molecular and biochemical toxicological research is presently conducted using *in vitro* models which vary in their ability to approximate toxic responses in the whole animal or human. Therefore, evaluation of the relevance of *in vitro* mechanistic findings, such as MAPK activation, to *in vivo* toxicity would lead to increased confidence in the use of mechanistic data in support of regulatory decision-making. This project will determine if changes in MAPK signaling studied *in vitro* can be used to extrapolate the MOA of chemical compounds *in vivo*. Research in project 3 will:

- Determine the effects of PM metals in MAPK activation *in vivo* and *in vitro*,
- Compare the responses to developmental toxicants in embryonic organ culture to those observed following *in vivo* exposure,
- Determine the role of integrin-mediated initiation of MAPK signal transduction *in vivo* in neurotoxic effects *in vitro* and *in vivo,*
- Conduct *in vitro* and *in vivo* effects of bioaccumulative chemicals such as the polychlorinated biphenyls (PCBs) on MAPK signaling in the developing nervous system, and
- Compare cell signaling in the nervous system *in vitro* and *in vivo* and determine it relationship to cognitive effects of environmental toxicants.

Project 4 - Extrapolation of Perturbations in MAPK Signaling from Animals to Humans. MAPK signaling cascades are highly conserved across species, indicative of the critical role these signaling pathways play in cell function. Due to this high degree of conservation, it is proposed that toxicants which perturb these pathways in one species will have qualitatively similar effects in the same tissue of another species. For the process of risk assessment, the uncertainty associated with extrapolation of data from animals to humans can be reduced if it is demonstrated that the MOA is the same in both species and if quantitative differences can be accounted for through data generated in tissues of humans and animal models. Project 4 will determine if effects of environmental toxicants on MAPK in one species will be qualitatively similar to those effects in other species. The studies outlined in this project will use several different *in vitro* models in which comparable rodent and human tissue can be obtained. These studies will also make use of the information gained in projects 1 and 2 in order to focus on the upstream targets, specific MAPK pathways, and critical biological effects important to the elaboration of pathogenesis for a particular model. Research covered by this project includes the following:

- Research to determine if MAPK signaling is perturbed by methylated trivalent arsenicals in mouse and human primary keratinocytes,
- Research to determine the effects of toxicants on MAPK signaling in human and rodent embryonic palatal cells, and
- Research to determine if alterations in cell signaling produced by neurotoxicants in rodent neurons are similar in human neurons.

Impact

A major goal of ORD's research program on human health is to develop a consistent, flexible set of principles for determining risk based on pertinent mechanistic data, regardless of the nature of the toxicity (i.e., harmonize cancer and non-cancer risk assessment approaches). There is an emerging knowledge base which suggests that the biochemical changes leading to certain cancer and non-cancer health effects are similar, and a more accurate prediction and characterization of risk for cancer and non-cancer endpoints can be based on understanding a chemical's MOA. In order to harmonize the risk assessment process for cancer and non-cancer health effects, information is needed regarding the application of mechanistic data for chemicals that produce different toxic outcomes by a similar MOA. The goal of this program project is to identify intracellular signaling pathways critical to the expression of carcinogenic and noncarcinogenic effects following chemical insult. This research will show that disruption of critical cell signaling pathways is a common MOA that underlies the toxicity of diverse groups of chemicals, that can be used as an *in vitro* predictor of the mechanism of toxic response *in vivo* and extrapolate from experimental animals to humans. This program project will provide an example of how data on common MOA can be used for comparative risk assessment across biological endpoints and also reduce the reliance on uncertainty factors by providing a common measure for extrapolation. In addition, the results of these studies may identify scientifically defensible biomarkers of exposure and effect.

Cross-Agency Interactions

As this program project progresses, consultations with scientists from NCEA will be held on a periodical basis in order to develop principles that can be used to formulate guidance documents on the harmonized use of MOA data in risk assessment.

Program Project 2: Disruption of Luteinizing Hormone (LH) Secretion as a Common MOA for Altered Fertility, Reproductive Disease, and Cancer of the Reproductive System

Objectives

- Characterize the range of environmental agents that alter LH secretion,
- Identify central nervous system (CNS) mechanisms involved in this MOA,
- Compare effects in animal models to those in humans, and
- Determine key events necessary for altered reproductive function, reproductive diseases and cancer of the reproductive system in the rodent and potentially the human**.**

Scientific Approach

One of the more difficult issues that confront risk assessors is the interpretation of toxicology data obtained in animal species and applying them to human risk assessment. A key to using such information in risk assessment is the degree of homology between humans and the test species; that is, whether there exists a common MOA. Within this context, the research in this program project is intended to derive a set of principles for application to animal data pertaining to toxicants that alter serum LH. This effort will focus on how LH disruption, in both the test species and humans, contributes to impaired fertility and reproductive disease. These principles can serve as a framework for evaluating the effect of environmental chemicals that alter the neuroendocrine control of LH. Moreover, as a valuable tool for examining test data, it will serve as a prototypical approach for examining dysregulation in other neuroendocrine axes of importance to human health risk assessment (i.e., prolactin and reproductive tumors; thyroid-stimulating hormone [TSH] and thyroid function; and adrenocorticotropin hormone and adrenal function).

Many xenobiotics have been shown to influence the regulation of LH. However, the actual mechanism (e.g., receptor, enzyme) may be different depending on the toxicant. Recently, alterations in LH secretion have been identified as the MOA for altered reproductive function (i.e., delayed puberty in both sexes, altered ovarian function in the adult female and pregnancy loss), premature reproductive aging, and mammary gland tumor development in the female following chlorotriazine treatment (Cooper et al., 1998, 1999, 2000; Laws et al., 2000; Narotsky et al., 2001; Stoker et al., 2002). Similarly, exposure to selected xenobiotics will produce changes in the secretion of LH in the male resulting in decreased testosterone; impaired fertility, or, as in the case of linuron exposure, elevated basal LH associated with an increased incidence of Leydig cell tumors. These effects of altered LH secretion generally require a protracted treatment before the adverse effect is observed. In contrast, it is important to note that all environmental compounds that modify LH secretion do not necessarily result in the same sequence of adverse reproductive or cancer effects. Thus, all toxicants that alter the LH surge (the MOA) may not lead to the same adverse outcomes. Based on these observations, the fundamental hypothesis to be examined herein is that, because the LH surge can be modified by environmental agents that interact with a variety of different CNS mechanisms, the adverse outcomes observed (in both rats and humans) will vary depending on which CNS target site is involved. Thus, a better understanding of the dynamics involved in the disruption of the LH surge will allow us to better predict which adverse outcome(s) will emerge and

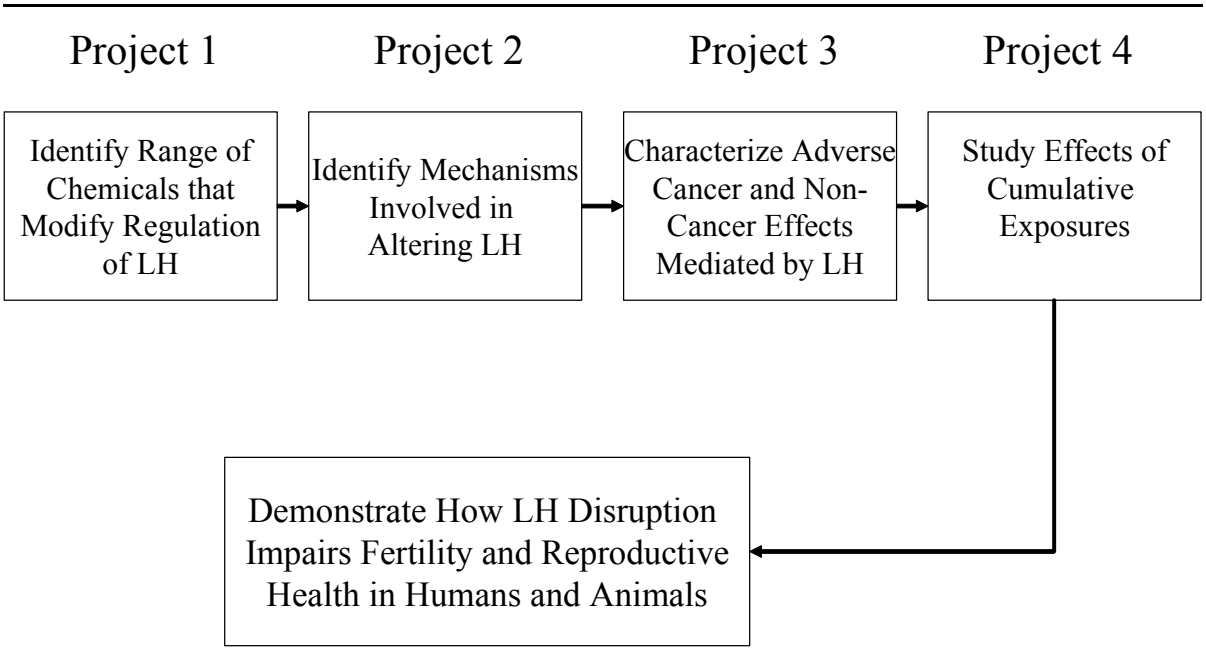

Figure 5 Critical Path for Research on Luteinizing Hormone

thus the relevance of each to potential human reproductive disorders can be better predicted. These studies will attempt to link the key cellular events responsible for changes in LH and adverse outcome in animals and humans, as summarized in Figure 5.

Project 1 - Identification of the Range of Chemicals that Modify the Regulation of LH. The purpose of this project is to determine the range of chemicals that may influence the secretion of LH. Once a chemical has tested positive in this evaluation, the dose- and time-response associated with oral dosing will be characterized using well-established techniques. These studies will provide important data describing the range of environmental chemicals that alter LH secretion, as well as the baseline information necessary for at least two of the projects described below. To date, several groups of environmental compounds have been shown to disrupt LH secretion in the female rat; however, the universe of chemicals that may possess such effects remains to be determined. To identify compounds for evaluation and testing, two different approaches have typically been used. First, those agents reported to affect any of the hypothalamic targets known to regulate gonadotrophin-releasing hormone (GnRH) and LH secretion would be predicted to affect LH in an ovariectomized, estrogen-primed rat model. Second, those compounds known to affect the estrogen or androgen receptor would test positive in assays using the intact female or male respectively. Either one or both models can be used to fully characterize the effect of the compound on LH. A third approach has been to identify compounds that, *in vivo*, are reported to cause adverse reproductive effects suggestive of LH disruption (i.e., altered cycling in the female rat, blockade of the LH surge, Leydig cell hyperplasia in the male).

Project 2 - Identify the Mechanisms Involved in Altering LH. The regulation of the LH surge is under the control of a variety of neurotransmitters and neuropeptides. Of particular interest is the fact that several xenobiotics affect noradrenergic and cholinergic

neurotransmission because both these neurotransmitters have been shown to regulate the pulsatile release of GnRH and subsequently LH secretion. Thus, compounds that alter noradrenergic (NE) synthesis (i.e., chlorotriazines [atrazine, simazine, propazine] and dithiocarbamates [thiram, metam sodium, disulfiram, carbon disulfide]), block NE receptors (i.e., chlordimeform, amitraz, etc.) or acetylcholine availability (i.e, carbamates) have all been reported to alter both basal LH secretion and the LH surge. Other environmental agents have been reported to alter the regulation of LH secretion, but the mechanism(s) remains to be determined.

The purpose of project 2 is to develop a database that documents the various mechanisms through which the regulation of LH secretion (both basal and pulsatile) may be altered. Should a compound be shown to alter the regulation of LH (project 1) by modifying pituitary or circulating concentrations, studies will be initiated to determine the particular mechanism of action underlying the observed effect. The particular approach(es) employed will depend upon published information about the compound in question, including available data about the compound's effects on other physiological processes using similar mechanisms of regulation and structure-activity (SAR) data available for other members of its chemical.

Project 3 - Characterization of Adverse Effects on Reproductive Function, Reproductive Diseases, and Cancers of the Reproductive System following Modification of LH Secretion. This research will use two approaches. The first approach addresses the hypothesis that environmental chemicals that change the timing or magnitude of LH release will alter reproductive function in both males and females. The purpose of these studies is to characterize the extent to which compounds that interfere with the regulation of LH alter a number of reproductive processes and to provide an approach that will form the basis for comparison between adverse reproductive outcomes in the rodent versus human. The second approach will address the hypothesis that long-term alterations in the endocrine milieu will lead to reproductive diseases and/or cancer. These studies will be dependent upon data obtained from studies described in the approach described above. For example, in cases where the chemical causes long term elevation of LH, additional endpoints will be included in the male and female pubertal studies to evaluate changes in ovarian and testicular histology. These studies will evaluate endpoints that are most relevant to potential human health concerns associated with this MOA.

Project 4 - Cumulative Exposures to Environmental Compounds which Suppress LH. There is concern that exposure to multiple chemicals may alter the effect in the target organ in ways not predicted or expected based on the dose-response curves of the single chemicals and an assumption with regard to additivity (i.e, toxicity may be greater or less than expected based on the single chemical information and an assumption of either dose addition or response addition). In addition, there is concern that cumulative exposure may cause toxicity in an unexpected organ. The default assumption for risk assessment of non-cancer endpoints is that toxic chemicals with similar MOAs will combine to cause toxicity in a dose-additive manner. However, there are relatively few data supporting this assumption, particularly for scenarios where toxic chemicals with similar MOAs, but different mechanisms will combine to cause toxicity. This scenario warrants further study. In this project, chemicals from pesticide classes which are identified in projects 1 and 2 as having the MOA in

suppressing LH will be evaluated in a dose-related manner. Once the MOA and portions of the specific mechanism within each general class are determined, low-dose cumulative exposure experiments will be designed. This project is linked directly with research on Cumulative Risk (see Section 5). All APMs for this project are reported in this Section on harmonization of the use of mechanistic data in risk assessment because the primary focus of the research is on identification and characterization of the LH MOA.

Impact

LH secretion is considered to be an important MOA for risk assessment, and chemical specific data will address key issues such as whether or not LH secretion is modified following exposure, the dose response, the pattern of change that occurs (chronic or acute disruption), the adverse outcomes that develop, and the mechanism involved. This information is critical for determining the potential health risks of exposure to the chemical in humans. By determining the key events involved in toxicant-induced chemical changes in LH secretion, a better understanding of the homology between the rat and human will evolve. This research will also provide significant information concerning the conditions underlying altered reproductive function and the role they may have in the development of tumors of the reproductive system (i.e., harmonization of cancer and non-cancer endpoints).

The work evaluating cumulative effects included in the current proposal will also address important questions concerning the evaluation of mixtures. There is a substantial degree of confusion within the Agency concerning how to select chemicals to be used in a cumulative risk assessments. The approach to be taken in this program project will be to link the cellular MOA responsible for the change in LH and adverse outcome. In these studies, the working hypothesis will be that compounds with similar mechanisms will show dose additivity; whereas combined exposure to compounds with different mechanisms would be either additive and potentially more than additive or less than additive.

Cross-Agency Interactions

The research efforts will involve ongoing communications with OPPTS. The effect of environmental agents on LH secretion and the demonstrated role of altered LH secretion in the development of adverse reproductive and cancer outcomes was of primary significance to the risk assessment of the chlorotriazine pesticides by OPPTS. These studies have also played a major role in the selection of compounds and the development of the cumulative risk assessment of the chlorotriazines again in close collaboration with personnel in OPPTS.

Program Project 3: Modulation of Cytochrome P-450s and Other Xenobiotic Metabolizing Enzymes (XME) Leading to Common MOA for Multiple Toxicities

Objectives

- Determine if P-450 and XME modulation is a common key event for multiple adverse effects in adult males,
- Determine if P-450 and XME is a common key event for multiple adverse effects in developmentally exposed males and females, and
- Develop computational toxicological approaches to evaluate if alterations in P-450 and/or XME are common key events for cancer and non-cancer effects.

Scientific Approach

This program project directly addresses the need to derive a commonly accepted set of principles defining how MOA information can be used in risk assessments, particularly as it relates to extrapolation issues. Evaluating whether chemicals have a common MOA for their multiple toxicities and the implications of that MOA for dose-response assessment are two of the fundamental problems in harmonization of human health risk assessment approaches. This project addresses both qualitative and quantitative methods that could be eventually applied to risk assessment activities.

Principles concerning the use of MOA data in risk assessment will be derived experimentally by examining a class of prototypic chemicals, the conazole fungicides (US EPA, 1999). Conazoles are used both as pesticides and pharmaceuticals; therefore, both experimental animal and human toxicity data are available. These fungicides are used in crop protection to control fungal infections on fruits, vegetables and cereal crops, and for seed treatment of cereal crops. Medically, they are used to treat local and systemic fungal infections. Conazoles are a class of ergosterol biosynthesis inhibiting fungicides (EBIFs) which inhibit 14-α-sterol demethylase (CYP51, lanosterol 14-α-demethylase). They contain either a 1,2,4-triazole or imidazole moeity which prevents enzymatic activity through interaction with the heme iron of P-450s. Because ergosterol is an essential component of fungal membranes, the inhibition of its biosynthesis leads to cell death. Conazoles have also been developed to selectively inhibit aromatase for the treatment of breast and prostate cancer. In vertebrate species, conazoles have complex effects on the hepatic and non-hepatic microsomal monooxygenase systems. They can act as both inducers and inhibitors of cytochrome P-450s depending on the tissue and specific conazole considered (Morita et al., 1990; Vinggaard et al., 2000).

Many conazoles are hepatotoxic and hepatocarcinogenic in mice. Some also induce thyroid follicular cell tumors in rats, and they are generally non-genotoxic using standardized test systems. Several conazoles are also hepatotoxic in humans. Both thyroid and liver cancer is thought to be mediated by modulation of P-450 or XMEs. Specifically, thyroid tumors are thought to be a result of increased hepatic metabolism and biliary excretion of thyroxine as the glucuronide leading to increases of TSH and overstimulation of the thyroid (Hurley, 1998). Hepatocarcinogenesis is thought to be a result of increased P-450 levels leading to oxidative stress, mitogenesis, and altered foci development ultimately leading to neoplasia.

In the liver, conazoles affect the activity and expression of a number of P-450s. For example, the pesticide propiconazole induces the activities of CYP1A1, CYP1A2, CYP2B1/2, CYP2B6, and CYP3A4. Additionally, propiconazole inhibits CYP2C11, and in reproductive and other tissues also inhibits CYP19 (aromatase). The pharmaceutical ketoconazole also induces several rat P-450s: CYP1A1; CYP2B and CYP3A2. Furthermore, ketoconazole inhibits the activities of CYP1A1, CYP1A2, CYP2A6, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 in the liver. In other tissues, ketoconazole inhibits essentially all the steroidogenic P-450s. Ketoconazole most potently inhibits CYP17, which is critical for two enzymatic steps in the synthesis of androgens in the testis. Ketoconazole also significantly affects male and female reproduction and is a potent developmental toxicant due to disruption of steroidogenesis.

Long-term regulation of steroidogenesis is primarily at the level of transcriptional regulation of the genes for the various steroidogenic P-450 enzymes. The P-450 genes are regulated in tissue-specific, developmentally programmed, and hormonally regulated fashions. There are substantial differences in the expression of these genes and the activity of their gene products among various mammals. Consequently, attention must be paid to significant species differences between humans and the rodent models. In the testis, LH modulates the production of testosterone in the Leydig cells, while follicle stimulating hormone (FSH) modulates estradiol synthesis (testosterone conversion by CYP19 [aromatase]) in the Sertoli cells. Both LH and FSH are secreted by the anterior pituitary, in response to the GnRH from the hypothalamus. Thus, monitoring LH and FSH will be critical in understanding conazole effects on steroidogenesis *in vivo* and subsequent effects and responses to altered steroid levels in the relevant reproductive, endocrine, and neural tissues. In addition to the sex steroids and the gonadalhypothalamic-pituitary axis, conazoles also affect glucocorticoid metabolism and the adrenal gland, thyroid metabolism and thyroid gland, and retinoid metabolism significant to embryogenesis. The critical path for this program project is summarized in the Figure 6.

Project 1- Profiling of Toxic Effects, P-450s, and Gene Expression in Multiple Tissues Following Conazole Exposures in Adult Male Rats and Mice. The purpose of this project is to evaluate the hypothesis that P-450/XME modulation is a common critical event that contributes to the MOA for hepatocarcinogenesis, thyroid carcinogenesis, reproductive toxicity, and neurotoxicity of selected conazoles in adult male rodents. It is hypothesized that the expression of numerous genes related to P-450 expression and function will be affected by conazole exposure in the various tissues being studied and that these altered expression profiles will provide mechanistic insights useful to identifying common MOAs. Research covered in this project will include the following:

- Establish dose-responses for pre-clinical and toxic effects in the liver, thyroid, testis and brain of young adult male Sprague-Dawley rats and CD-1 mice following 14 daily doses by gavage with various conazoles;
- Delineate conazole effects on P-450 protein expression and activity in liver, thyroid, testis, and brain and evaluate tissue dosimetry in these and selected other tissues; and
- Profile gene expression of P-450s and XMEs in testis, brain, thyroid, and liver of mice exposed to conazoles.

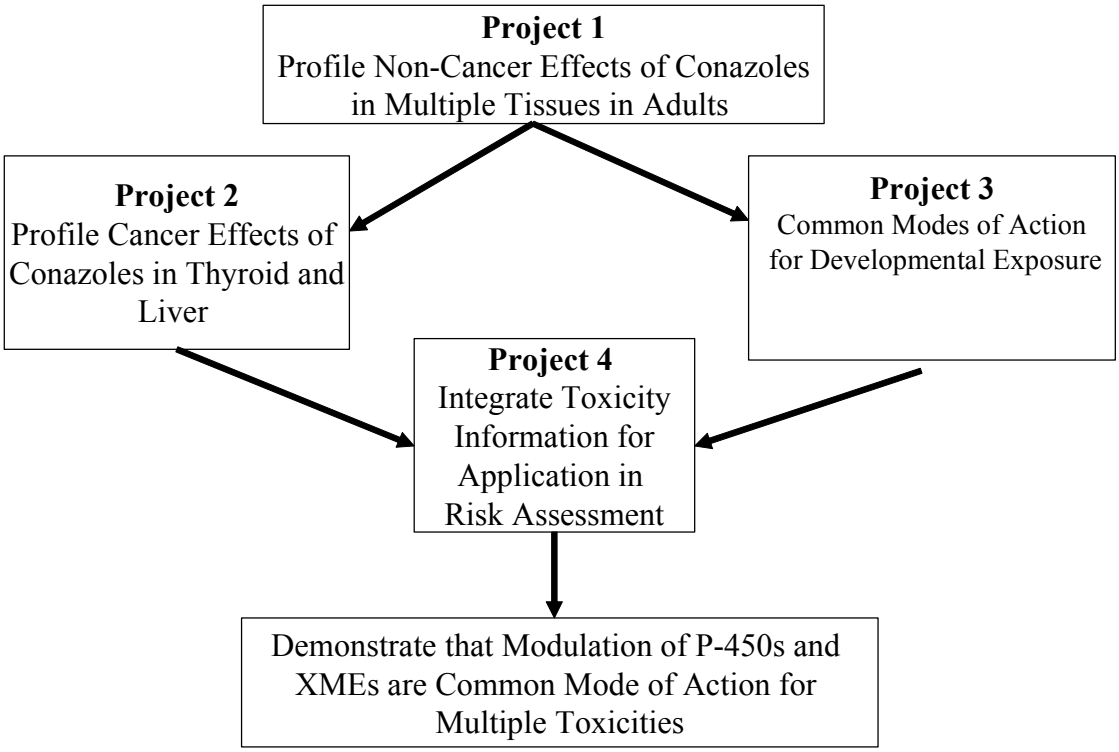

Figure 6 Critical Path for Research on P-450 and XMEs

Project 2 - Adult Exposures Resulting in Thyroid and Liver Cancer. This research will determine the effects of conazole in thyroid and liver of rats exposed during adulthood and will consist of the following studies:

- Research on the MOAs of conazoles as both thyroid hormone disruptors or as progenitors of toxic intermediates with both MOAs occurring through conazole-induced enhanced metabolism, and
- Studies to examine the early events in conazole-induced hepatic neoplasia and relate these findings to P-450 induction to exclude genotoxic intermediate involvement as a result of conazole-induced P-450 metabolism.

Project 3- Determining Common MOAs for Reproductive and Neural Toxicities Following Developmental Exposure to Conazoles. Developmental effects are expected at various stages of growth as a result of both gestational and postnatal exposures to conazoles. It is possible that differential responses could be observed depending on age, gender, or physiological status. The goal of this research is to understand how common MOAs produce a range of toxicities in developing tissues, following exposure to conazole fungicides. These studies will focus upon modulation of cytochrome P-450s and other xenobiotic metabolizing enzymes to identify common MOAs, but will also assess broader effects on gene expression. The general hypothesis is that disruption of steroidogenesis by conazoles in developing neural, endocrine, and reproductive tissues will result in adverse effects at lower doses than in adult

animals. Furthermore, because this common MOA is shared across various tissues, mechanistic data resulting from P-450 and gene expression profiling will be useful in developing new approaches for harmonizing risk assessments across various toxicities. These approaches should be especially relevant and applicable in assessing the risk of environmental exposures for children. Research covered by this project will evaluate the following:

- Effects of gestational exposure to conazoles on gestational hormone levels, gestation length, pregnancy maintenance and pregnancy outcome;
- Effects of gestational exposure to conazoles on female fetal development;
- Effects of gestational and postnatal exposure to conazoles on timing of female puberty and adult reproductive function;
- Effects of gestational exposure to conazoles on male fetal development;
- Effects of gestational and postnatal exposure to conazoles on timing of male puberty and adult reproductive function;
- Effect of gestational and postnatal exposure to conazoles on the developing neurological system in both male and female rats;
- Tissue dosimetry of parent compound (and potentially active metabolites) to assist in interpretation of results of toxicity studies; and
- Selected cancer-related biomarkers in rats developmentally exposed for 90 days for comparison with 90-day exposures in young adults (project 2).

Project 4 - Computational Toxicology: Integrating Toxic Effects, P-450 Modulation, SAR Analysis, and Gene Expression pRofiling for Application to Risk Assessments. The term computational toxicology will be broadly employed in the context of this project to encompass a variety of techniques and approaches for accessing and modeling existing and newly generated data on the conazoles, their varied biological responses, and the activities of structurally and biologically related chemicals. The ultimate aim is to derive useful generalizations with respect to chemical structure, gene expression patterns, and biological activation mechanisms that can inform and harmonize future evaluation of conazoles as well as, perhaps, other chemicals with P-450-mediated toxicities and common MOA characteristics.

The operating hypothesis is that shared structural features and physicochemical properties, as well as common patterns of gene expression, can serve as useful organizational principles and potential predictors of metabolic and biological response. More specifically, characteristic profiles of structural/biological response could suggest appropriate chemical analogues, narrow the consideration of potential adverse effects and animal models, and aid in the design of a rational testing strategy for new chemicals. Research covered by project 4 will involve the following:

- Provide insight into the structural basis for the P-450-mediated toxicological effects of conazoles and related compounds from survey of environmental and pharmaceutical data sources and use of SAR models;
- Use of computational informatic approaches for analyzing gene expression profiles of P-450-mediated toxicities across multiple test systems (chemicals, species, tissues, doses)

towards the goal of harmonizing risk assessments; and
- Utilization of relational database searching strategies to explore broader associations between P-450 expression and activity, gene expression profiles, chemical structure, and biological effects to provide guidance in future assessments of conazole-like chemicals and P-450-mediated toxicities.

Impact

This program project directly addresses the need to derive a commonly accepted set of principles defining how MOA information can be used in risk assessments, particularly as it relates to extrapolation issues. Evaluating whether chemicals have a common MOA for their multiple toxicities and the implications of that MOA for dose-response assessment are two of the fundamental challenges in harmonizing human health risk assessment approaches. This project addresses both qualitative and quantitative methods that could eventually be applied to risk assessment activities.

Cross-Agency Interactions

From its inception, this project has been discussed and modified based on numerous meetings with the Office of Pesticide Programs (OPP). OPP has provided critical insight into chemical selection, dosing and bioassay endpoints, throughout the project's development. We have already planned periodic meetings with OPP during the conduct of this project to keep them informed about progress as well as to garner advice on future studies.

3.5 Gap Analysis and Links to Other Multi-Year Plans

NHEERL research on the harmonization of risk assessment will help identify principles concerning the use of mechanistic data in cancer and non-cancer risk assessment through the study of common MOAs and key biological events associated with three specific toxicity pathways, i.e., cell signaling pathways, alterations of LH secretion, and modulation of P-450 and XMEs. Because these are only several of the many toxicity pathways that would be of relevance to risk assessors, this may seem to be a limitation. However, the pathways selected are relevant to a large number of environmental pollutants. It should also be noted that the mechanistic research described in this implementation plan has significant links to several ORD MYPs (e.g., Air Toxics, Drinking Water, Endocrine-Disrupting Chemicals [EDCs]). Information from all of these areas will contribute to the overall goal of developing a framework for the use of mechanistic data in risk assessment (see Figure 7).

For example, research on EDCs focuses on mechanisms of estrogenic, androgenic, and thyroid-mediated effects. EDC research will characterize the effects of exposure to multiple EDCs in various combinations such as those with similar and different MOAs and will determine the degree to which effects of EDCs with defined MOAs can be extrapolated across chemical class. EDC research to develop standardized protocols for screening chemicals for their potential endocrine-mediated effects is also based on an understanding of potential mechanisms of MOAs underlying the tests. Thus, research on EDCs will inform discussions on the identification of potential common modes of action and will provide supporting data with which to develop a framework concerning the use of mechanistic

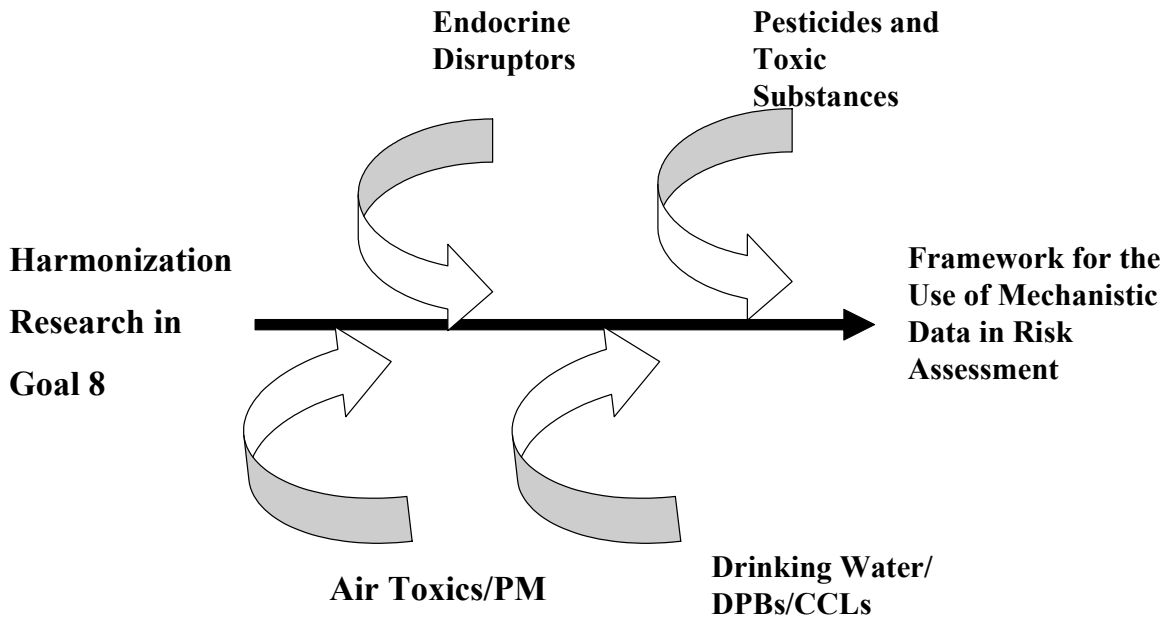

Figure 7 Framework for Use of Mechanistic Data

information in risk assessment. Furthermore, emerging technologies involving the use of proteomic and genomic techniques are used to develop sensitive screens and tests for EDCs and will complement harmonization research to determine the utility of emerging technologies.

In the area of drinking water, much of the research on (As) involves elucidation of the MOA of cancer and non-cancer effects and the development of PK models based on mechanistic studies. The results from these studies will complement harmonization research on other potential common MOAs with other chemicals (i.e., cell signaling, oxidative stress, hormonally-mediated effects). Harmonization research will also benefit from work on the mechanisms of carcinogenicity of priority disinfection by-products (DBPs), an evaluation of the scientific basis for common MOAs of DBPs, and their application to risk assessment. Research to provide a sound scientific basis for the development of Contaminant Chemical List (CCL) #3 will also provide mechanistic data on cancer and non-cancer endpoints for selected CCL contaminants. Finally, research to improve data and tools for assessing and managing potential health risks associated with drinking water contaminants will involve a development of a weight-of-evidence approach based on common mechanisms and toxicity for cumulative risk assessment for drinking water contaminants. Thus, mechanistic data from research on several drinking water contaminants will provide significant input into the development of a framework for the use of mechanistic data in risk assessment.

Research on Air Toxics involves the development of mechanistic data to support risk assessment, including research to estimate human health effects and aggregate exposures to hazardous air pollutants (HAPs), to extrapolate animal-to-human data for selected HAPs, to determine the shape at low doses of the dose response curve, and to develop models for characterizing and predicting toxicity of selected air pollutants and mixtures based on MOA information. Harmonization research to develop a framework for the use of mechanistic data in risk assessment will also

benefit from mechanistic work to identify the mechanisms of toxicity for PM constituents and/or its sources. MOA research on potential common MOAs of pesticides and toxic substances will further the development of a framework for use of mechanistic data in risk assessment.

Research on the use of mechanistic data in risk assessment will also significantly affect the other themes described in this implementation plan, i.e., susceptible subpopulatons and cumulative risk. For example, risk assessment for cumulative risk (see Section 5) is based on the risk of combined toxic effects of chemicals with similar or dissimilar MOAs. Mechanistic research to identify common MOAs and to develop emerging technologies will be crucial to developing the tools needed to assess cumulative risks associated with exposure to multiple chemicals, mixtures, and other factors across the Source-to-Outcome Continuum. Research on common MOAs using prototypic chemicals to provide the scientific basis for the use of mechanistic data in risk assessment will also identify key events in specific toxicity pathways that can be used to develop framework and protocols for assessing cumulative exposures and risks and to develop methods and measurement data that will support models of cumulative exposures, dose, and effects.

Much of the research on susceptible subpopulations (see Section 4) focuses on the biological basis for differential responsiveness to chemical exposures. A crucial factor that must be determined is whether the MOA of a chemical is the same in the susceptible subpopulation as in the general population. For example, to evaluate the risks from childhood exposure, it would be important to know if PK factors resulted in an increased concentration of the active chemical at the target site or if key events in the toxicity pathway are of increased frequency or expressed at a higher rate in the susceptible subpopulation. It is also possible that infants or children may have differential sensitivity because a chemical acts on a developmental process that is not present in the adult. Harmonization research on potential common MOAs and identification of key events in representative toxicity pathways from research on harmonization will be important in determining the variation in susceptibility to environmental agents as a result of health status, aging, and genetic factors. This research will also evaluate the relationship between adverse health outcomes and exposure to environmental agents *in utero* and during infancy and childhood and will elucidate the role of environmental agents in the induction and exacerbation of preexisting diseases such as asthma.

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Section 4
Susceptible Subpopulations

4.1 Problem

The variability in responsiveness of humans to environmental pollutants can be associated with differences in biological susceptibility. As discussed in the ORD *Human Health Research Strategy,* variation in susceptibility depends on intrinsic factors such as life stage and genetic factors, as well as acquired factors such as disease. With regard to life stage, there are specific periods or windows of vulnerability during development, particularly during early gestation, but also throughout pregnancy and early childhood through adolescence when toxicants might permanently alter the morphology and/or function of an organ system. Children may also be more vulnerable to specific environmental pollutants because of differences in absorption, metabolism, and excretion. In addition, children's exposures to environmental pollutants are often different from those of adults because of different diets and different activities (e.g., playing on floors and in soil and mouthing of their hands, toys, and other objects) that can bring them into greater contact with environmental pollutants. The effect of aging on response to environmental exposures is another area of uncertainty. Older adults respond differently than younger adults to environmental exposures because of age-related changes that limit the body's ability to maintain homeostasis and respond to injury. Research at NHEERL will examine the effect of aging on responses to environmental pollutants and will develop predictive models that can be incorporated into the risk assessment process.

The genetic factors that could predispose human subpopulations to adverse effects from exposure to pollutants include genetic polymorphisms for metabolizing enzymes, differing rates of DNA repair, and different rates of compensation following toxic insult. The main scientific question for this research is whether such genetic differences significantly influence risk at realistic, low dose exposures. Information on gene-pollutant interactions as a result of long-term exposure to environmentally relevant concentrations of pollutants will be explored.

Preexisting diseases may influence the response to environmental toxicants by altering xenobiotic metabolism or otherwise altering the host's response in a synergistic, additive, or antagonistic manner. ORD research has shown, for example, that mice challenged with influenza have increased mortality from exposure to several environmental agents including dioxin, ozone, and ultraviolet radiation. NHEERL research will develop animal models of disease having a high incidence in the human population (e.g., asthma) and will determine the effects of the disease on the dose-response curves for high priority environmental agents (e.g., air pollutants).

4.2 Goals

The overarching goal of NHEERL research on susceptible subpopulations is to identify the biological basis underlying differential responsiveness of susceptible subpopulations of humans to pollutant exposure. This implementation plan will address the following issues:

- Identifying the adverse effects of susceptible subpopulations that are qualitatively or quantitatively different from effects in the larger population,
- Determining the PK and PD basis for differential responsiveness of susceptible subpopulations to environmental agents,

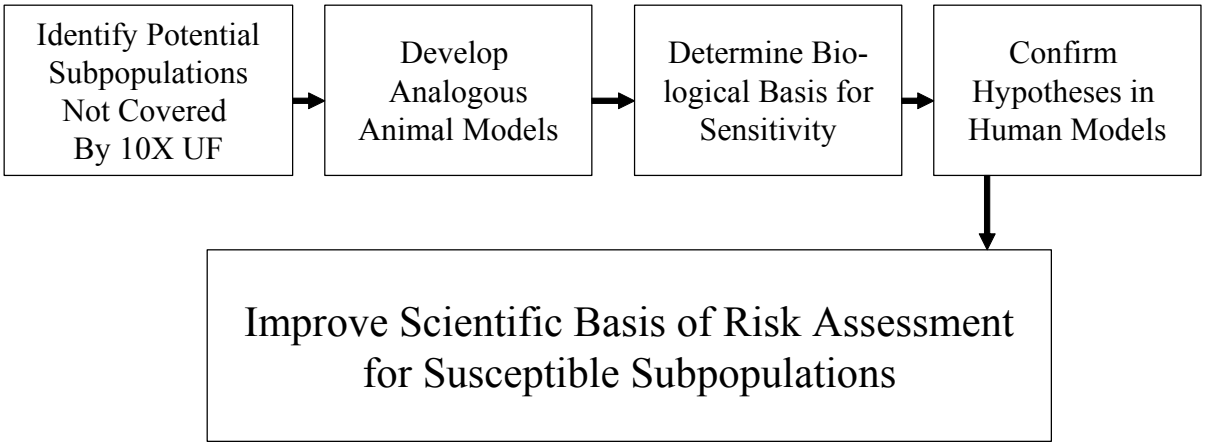

Figure 8 Critical Path for Research on Susceptible Subpopulations

- Developing methods and models in animals that can be used to predict responses in human susceptible subpopulations,
- Determining the relationship between exposures earlier in life to adverse health effects later in life,
- Determine the influence of critical periods of development on expression of toxicity, and
- Determining the quantitative contribution of genetic polymorphisms to responsiveness to environmental agents.

4.3 Critical Path

Research to improve the scientific basis for risk assessment of susceptible subpopulations will follow the steps outlined in Figure 8. Research must first identify which subpopulations in the general population may not be protected by current risk assessment methodologies. For example, in the case of non-cancer risk assessments, an uncertainty factor of 10 is used by risk assessors to account for variability within the general population. Research has indicated that this factor, under some circumstances, may not be fully protective of children's health. NHEERL research will continue to focus on infants and children as a susceptible subpopulation while exploring the relative sensitivity of other subpopulations, especially older adults, those with preexisting diseases such as asthma, and those with specific genetic polymorphisms. Once a potentially susceptible subpopulation has been identified, steps will be taken to develop animal models that are as analogous as possible to the human situation. This step is important to ensure that the results from animal studies can be extrapolated to humans and will reduce uncertainties in the risk assessment process. Appropriate animal models will also be used to investigate the underlying PK and PD differences between the susceptible subpopulation and the general population. Results from animal studies will generate hypotheses that can be tested in human epidemiological studies, and results from human observational studies will be examined in animal models to help determine susceptibility factors and underlying MOAs.

NHEERL research on susceptible subpopulations will be consistent with the APGs and APMs for Susceptible Subpopulations identified in the ORD *Human Health Research Multi-Year Plan* (Appendix C).

4.4 Program Projects

Four program projects address life stage issues. Program project "Identifying and Validating Biologic Indicators of Susceptibility and Sensitivity among Children to Assess Potential Risk of Adverse Health Outcomes" is concerned with identifying why children are differentially susceptible to environmental pollutants. Program project "Extrapolating Across Windows of Vulnerability to Assess Children's Health Risks Using Rodent Toxicity Data" focuses on how exposure, dose, and effect information can be incorporated into risk assessment methods to account for interindividual variability. Program project "Long-Term Effects of the Developmental Environment" addresses the long-term consequences of perturbations during the *in utero* period and various diseases later in life. Program project "The Aged as a Susceptible Subpopulation" focuses on the differential sensitivity of the older adults to environmental exposures.

The program project "Environmental Risk Factors for Asthma" focuses on environmental influences that act directly as allergens to induce asthma or agents that enhance either the induction or exacerbation of the disease via non-specific stimulation of immune or inflammatory responses. Two program projects address genetic components that may predispose subpopulations to exposure to environmental agents. The program project "Oxidative Stress as a Common Susceptibility Attribute for Non-Cancer Risks" focuses on the role of underlying oxidative stress as a common susceptibility factor guiding response to pulmonary, cardiovascular, neural and reproductive toxicants. Program project "Genotype and Phenotype in the Metabolism, Toxicity and Carcinogenicity of Arsenic" focuses on genetic capacity of individuals to metabolize inorganic arsenic (iAs) as a primary determinant in susceptibility.

Program Project 4: Identifying and Validating Biologic Indicators of Susceptibility and Sensitivity among Children to Assess Potential Risk of Adverse Health Outcomes Associated with Environmental Exposure

Objectives

- Identify and validate comparable biologic indicators in both test animals and humans that can be used to investigate the risk of environmental exposures associated with cancer and functional impairments of the nervous, immune, and reproductive systems; and
- Identify genetic and physiologic factors that can modify the associations between exposures and outcomes in a similar fashion in both animal and humans.

Scientific Approach

There is substantial public health concern that variation in the quality of the environment causes adverse health outcomes in children. Many causes of childhood mortality, disease, and disabilities affecting quality of life are plausibly associated with environmental factors. In order to better inform policy and risk assessment, multifaceted scientific studies are needed to provide bioindicators which help identify environmental factors that are harmful, harmless, or helpful in terms of their effects on children's health and development. The development and validation of biomarkers that are applicable across species are vital to improve the precision of risk estimates. Thus, the major unifying theme for this program project is identifying and validating biological indicators of susceptibility and sensitivity to assess the potential risk of adverse child health outcomes associated with environmental exposures.

This program project will assess a broad range of potential biomarkers. For example, neurodevelopmental biomarkers will focus on neurotrophic factors (NTFs), neurotransmitters, proinflammatory cytokines, and classical conditioned responses. Research on immunological biomarkers will assess antibodies, cytokine proteins, message analyses, and phenotypic lymphocyte profiles. Reproductive function will be assessed using mRNA and protein expression analyses, immunoglobulins, proteins and other markers. Cancer risk will be examined by looking at underlying mechanisms which might account for inter-individual phenotypic variation in response measured by a wide range of health indicators. All projects in this program project will focus on maximizing animal-to-human extrapolation by identifying and validating common biomarkers with a focus on samples that could be obtained non-invasively from human subjects. While human studies will be generally observational with respect to exposure assessment, animal *in vivo* studies and *in vitro* studies allow for direct manipulation of exposure and evaluation of biological indicators that are predictive of adverse effects. This interactive and iterative process between animal and human studies will allow for the anticipation of the types of health effects that might be expected based on specific exposures and gives important clues about the underlying MOAs for complex disease-exposure relationships.

The research in this program project is linked to the National Children's Study (NCS), a multi-agency, multi-disciplinary longitudinal study designed to evaluate children's health in the United States (US). Under the Children's Health Act of 2000, the Agency will work with other Federal Agencies to plan and implement the NCS.

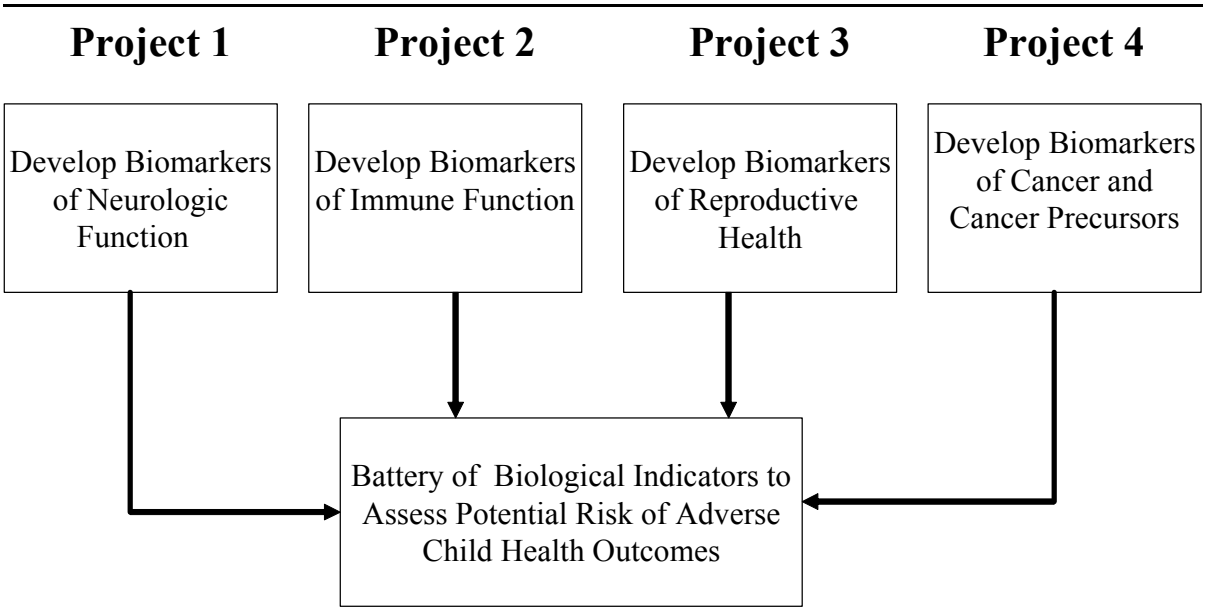

Figure 9 Critical Milestones for Research on Children's Health

Research at NHEERL is designed to coordinate animal and human studies that focus on endpoints where the burden of illness, disability, or death is high for children in the US and where an environmental factor is implicated in the disease process. One of the unique strengths of NHEERL is the ability to coordinate human and epidemiological studies with toxicological studies in test animals. This research program will influence the selection of biomarkers assessed in the NCS. The critical steps to be followed in this program project are summarized in Figure 9.

Project 1 - Biomarkers of Neurologic Function in Children. Neurological and developmental disorders in children are a major public health concern. The etiology of most mental retardation and many other neurodevelopmental disorders is unknown. Autism spectrum disorder may be increasing in prevalence, and some neuropsychologic conditions of childhood such as attention deficit-hyperactivity disorder (ADHD) are being diagnosed at an epidemic rate. Environmental exposures to known or suspected neurotoxicants have been implicated in these disorders of children. In order to better understand the mechanisms that link exposures to neurodevelopmental outcomes in children, biomarkers in test animals that can be applied to human populations will be developed. Effects that are observed in human populations will be more fully evaluated by characterizing dose-response relationships using animal models (Barone et al., 1998; Lassiter et al., 2002; White et al., 2002).

One series of studies will attempt to demonstrate the association and predictive validity of early postnatal serum measures of NTFs and neurotransmitters with chemical-induced brain damage in rats with the goal of validating comparable early postnatal serum measures with autism spectrum disorders, ADHD, Downs Syndrome, and cerebral palsy. The hypothesis for this work is that alterations in expression profiles of NTFs, neuropetides, and cytokines are predictive for developmental disorders and functional impairments of the nervous systems. These studies will:

- Evaluate the association and predictive validity of early postnatal serum measures of NTFs, cytokines, and neurotransmitters with chemically-induced brain damage in animal models;
- Assess the feasibility of measuring early postnatal serum measures of NTFs, neurotransmitters, and cytokines in human infants; and
- Assess the feasibility of determining the predictive validity of early postnatal serum measures of NTFs, neurotransmitters, and cytokines in human infants with developmental neurological disorders.

A second series of studies will focus on developing analogous methods to evaluate neurobehavioral function and animal models. The hypothesis for this research is that developmental disorders in humans are linked to fundamental processes that are common to humans and experimental animals and can be quantified. These studies will:

- Assess the feasibility of a field ready test system based on classical conditioning (e.g., eye blink conditioning) for use in human infants;
- Investigate other potential measures of neurobehavior (e.g., signal detection, reaction time) applicable in both humans and experimental animals;
- Establish the ontogeny of motor function in an animal model ;
- Determine whether early (prenatal, postnatal and/or perinatal) exposure to toxicants alters the ontogeny of motor function in animals and humans;
- Establish a conditioned-behavioral test for quantifying susceptibility in animals and humans; and
- Determine whether early exposure to toxicants alters awareness and sensitivity to the consequences of behavior in animals.

Project 2 - Biomarkers of Immune Function, Developing Animal Models that Predict Immune Function in Human Children. Xenobiotic exposure during critical windows of immune system development can cause alterations in function that persist into adulthood, and possibly for life (Weisglas-Kuperus et al., 2000). Evidence obtained in experimental animals strongly suggests that the developing immune system is much more susceptible than that of the adult. This project will test the hypothesis that results of standard clinical immunological assays (e.g., antibody responses, cytokine protein and message analysis, and flow cytometric phenotyping of lymphocyte subsets) are useful predictors of changes in host immunocompetence and provide detailed information on the specific response to antigens or vaccines. Included in the definition of xenobiotics are environmental microorganisms and infectious agents; thus, the influence of early-life infection on immunocompetence (both positive and negative) will be evaluated. The results may provide useful markers for increased susceptibility to infectious disease and may predict the loss of homeostatic control that may lead to the development of immune suppression, allergy, or autoimmune disease. Many disorders of childhood have an immune function component and some, e.g., asthma, are increasing and suspected to have environmental antecedents. The hypothesis for this work is that xenobiotic exposure during critical windows of immune system development will alter immunological function that persist into adulthood and possibly for life**.** Research in project 2 will

- Evaluate the effects of developmental exposure to pesticides and assess the predictive value of the animal model for responses of children exposed to environmental chemicals,

- Assess the effects of developmental exposure to endocrine-active xenobiotics on immune function in animals, and
- Determine MOA of rodent developmental immunotoxicants as a means to identify candidate markers that reflect possible effects in exposed humans.

Project 3 - Biomarkers Related to Reproductive Health Risks. Environmental exposures during preconception and early pregnancy can affect fertility, outcome of pregnancy, and the health and development of children from the pregnancy. Two approaches will be used to evaluate reproductive health risks. One uses samples obtained with minimal invasiveness (e.g., hair, milk, and blood) that may be accessible for collection from human subjects and compares the results between effects on surrogate and target-tissue (e.g., gonads) markers of exposure and effect. The second approach looks at the placenta to help identify useful biomarkers related to the interface between the outside environment of the mother and the developing fetus. Both approaches are important for predicting the eventual health of the offspring. In addition to biomarkers that may be useful to predict the offspring's eventual reproductive capacity, biomarkers that predict reproductive success in the parents also have predictive value for child health outcomes. Parents with suboptimal fertility (such as those with a long time-to-pregnancy) are at higher risk for adverse pregnancy outcomes including low birth weight and preterm delivery. The biomarkers developed in this project may have value in measuring children's risk associated with their parents reproductive capacity as well as predicting the child's reproductive health. The hypothesis for this project is that gene expression profiling of reproductive tissues can reveal genetic biomarkers of exposure and effect for reproductive toxicants. Studies included in project 3 are as follows:

- Evaluating the best methods to collect, store and transport surrogate tissues (blood, hair follicles, uroepithelial cells, and semen) from humans and rodent models such that sufficient quantities of good quality RNA can be extracted for gene expression analysis or identification purposes, respectively;
- Confirming that historical, archived placental specimens have sufficient macromolecular integrity for utilization in mechanistic/biomarker studies;
- Exposing fetal, juvenile, and adult rats and mice to effective doses of known and suspected reproductive toxicants (i.e., disruptors of steroidogenesis such as conazole fungicides) and using gene expression analysis of target tissues (testis, prostate, ovary, uterus) to identify genetic biomarkers;
- Determining whether gene or protein expression profiling in surrogate tissues can provide reliable biomarkers for exposure to reproductive toxicants (e.g., conazoles, PCBs, chloroatrazines) in adult humans; and
- Determining whether gene expression profiling in surrogate tissues from children (blood, hair follicles, uroepithelial cells) is possible using the same methods and genes identified in the fetal/juvenile rodents and adult humans.

Project 4- Biomarkers of Cancer and Cancer Precursors: Identification of Genetic Susceptibility, Biomarkers of Gene Expression, and Oxidant Status. Most suspected associations between environmental exposures and adverse health outcomes for children are likely to have a component of gene-environment interaction or variations in susceptibility. These variations in susceptibility make it difficult to assess risk in the general population because we are unable to effectively identify any at-risk subgroups. Consequently, the heterogeneous risk pool

dilutes risk estimates and drives them towards the null. The aims of this project are designed to enhance our ability to determine the important genetic factors involved in gene-environment interactions and to assess potential co-factors such as reactive oxygen species (ROS) and antioxidant status and how they relate to disease. The capacity ability to measure similar processes in animal and human studies will advance our ability to determine which genetic and physiologic factors are most important in assessing children's environmental health risk. Research in project 4 will:

- Evaluate whether dose, individual and age-specific (adult vs children) differences in sensitivity to chemicals and mixtures can be assessed in human blood, plasma, and/or individual blood cells following *in vitro* treatment to environmental agents;
- Examine various housekeeping genes and their functional products, such as those associated with DNA repair, cell cycle control and apoptosis, to determine if they respond differently to chemical stressors in a manner that is dependent on the life stage;
- Assess the utility of breast milk epithelium and blood cells for determining the contribution of genetic variability (expression profiles and polymorphisms) on an individual's sensitivity to environmental toxicants;
- Evaluate the extent to which plasma concentrations of antioxidants (ascorbate, urate, tocopherols and glutathione) are cofactors that might modulate risk factors in the initiation of diseases in children;
- Validate use of human blood *ex vivo* in the evaluation of dose, genetic, and age-specific factors that affect inter- and intra-individual phenotypic disease measurements; and
- Develop reliable assays to measure endogenous and exogenous constituents of breast milk that are implicated in altering health status of women or their breast-fed children.

Impact

Under the Children's Health Act of 2000, the Agency is working together with other federal partners to plan and implement the NCS. Within ORD, the importance of research on children's environmental health is a long-standing priority with critical implications for risk assessment. This program project will demonstrate scientific leadership in children's environmental health research by coordinating animal and human studies that focus on endpoints where the burden of illness, disability or death is high for children in the US and where an environmental factor is implicated in the disease process. One of the unique strengths of this implementation plan is the ability to coordinate human and epidemiologic studies with toxicology studies in test animals. This research program can influence the selection of biomarkers assessed in the NCS, which will be the major study of children's environmental health of this generation.

Cross-Agency Interactions

This program project benefits from substantial interaction and coordination with Agency efforts in planning the NCS. In cooperation with several other federal agencies, under the leadership of the director of the National Institute for Child Health and Human Development, the Agency has a strong role in developing this major child environmental health study. The Agency representatives to the Interagency Coordinating Committee (ICC) of the NCS are supportive of this program project and maintain contact with the investigators to insure that the biomarkers evaluated and

validated will be of optimum utility. Members of the ICC come from across the National Institutes of Health (NIH), Centers for Disease Control and Prevention (CDC), and the Agency. The Agency representatives are from NHEERL, NERL, and NCEA. This proposed research plan has been reviewed by members of the ICC and the lines of open communication are insured by the NCS web-based portal (a copy of this research proposal and updates are maintained on the portal for ready access by study planners). The study planners also interact regularly with NCER regarding extramural funding for NCS projects. This program project is also integrated with a series of Agency-funded pilot studies for the NCS which brings together investigators from across ORD to support method development for the NCS.

Program Project 5: Extrapolating Across Windows of Vulnerability to Assess Children's Health Risks Using Rodent Toxicity Data

Objectives

- Focus modeling and laboratory research around selected case studies including: volatile organics (including methanol), organophosphate (OP) pesticides, and conazoles;
- Obtain physiological, biochemical, and anatomical parameters for rodents and humans for selected windows of vulnerability;
- Quantify metabolism, and perhaps other PK processes such as absorption in humans and rodents for the selected windows of vulnerability with *in vitro* methods;
- Develop physiologically based PBPK models and generate rodent PK data for the case study chemicals during the windows of vulnerability in rodents and humans;
- Develop human exposure models during the windows of vulnerability to link to the PBPK models; and
- Delineate the magnitude of age-related differences in sensitivity to pesticides and elucidate mechanisms (kinetic and/or dynamic) underlying this difference in sensitivity.

Scientific Approach

There are windows of vulnerability that exist during various life stages during which exposure to environmental chemicals may result in permanent damage to biological systems. These windows may exist at various stages of gestation, as well as postnatally, during infancy, childhood, or adolescence. One major source of uncertainty and potential source of error in the assessment of the health risk of children derives from developmental events occurring in different time periods for different species. This is particularly apparent for events that occur in the early postnatal (lactational) period for rodents and *in utero* for humans. Thus, lipophilic chemicals such as PCBs, which disrupt thyroid hormones, cause developmental hearing toxicity in rodents during the lactational period due to massive mobilization from the mother's fat reserves to her milk during lactation. This same effect would not likely occur following postnatal exposure in humans because hearing development occurs *in utero*. On the other hand, the situation would be reversed if a chemical were preferentially accumulated in the fetus. Clearly, extrapolations between animals and humans will be dependent upon the developmental processes at risk. Because animal toxicity data will continue to play an important role in the assessment of a chemical's potential to affect children's health, there is a need to develop a systematic approach to assess the relevance of animal toxicity data occurring during different developmental periods, both prenatal and postnatal, to humans and to extrapolate the response from animals to humans. It will be necessary to first identify critical periods of developmental processes in controls before beginning studies using very specific chemicals with known MOAs. The critical steps for research in this program project are summarized in Figure 10.

Project 1 - Literature Review. The main objective of this effort is develop a framework that includes exposure scenarios and PK processes associated with different windows of vulnerability to assess children's health risk from animal toxicity data. The following issues will be addressed by the literature review:

- Physiological parameters for absorption, distribution, metabolism and elimination of selected environmental chemicals will

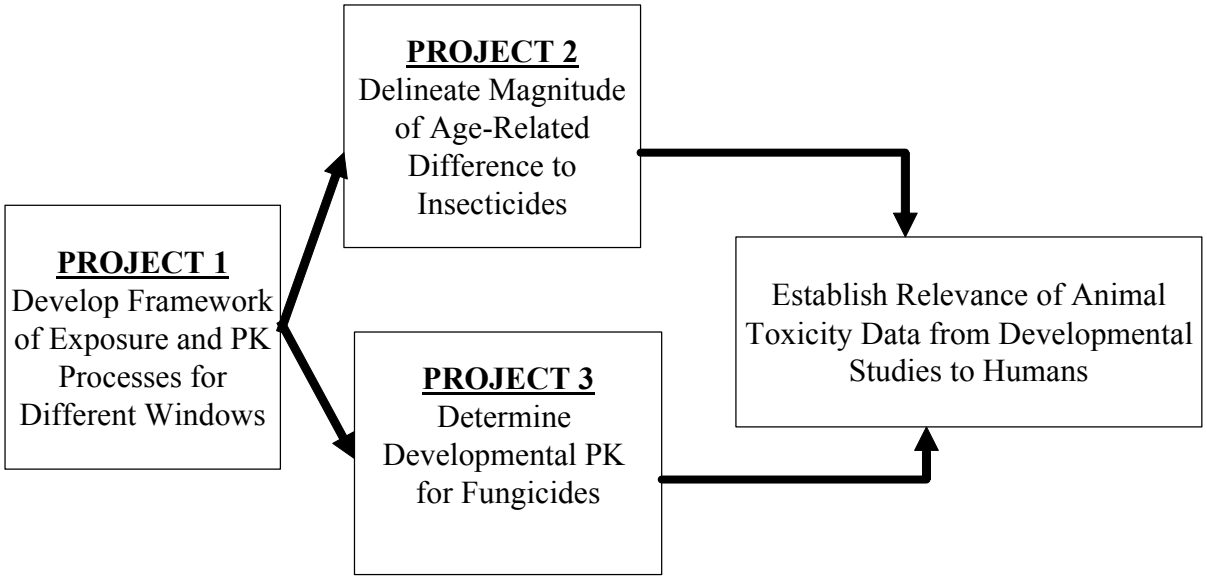

Figure 10 Critical Path for Research on Windows of Vulnerability

be obtained for several animal species *in vitro* PK parameters will be determined in order to:

- identify the enzymes involved in chemical metabolism and to quantify their expression in adults and children when that variance may contribute to susceptibility to toxic injury and
- develop chemical-specific *in vitro*-derived measures of tissue distribution of parent chemical and toxicologically-active metabolites.

- Develop PBPK models and rodent PK information for various life stages. Methanol was selected due to ongoing risk assessment activities in NCEA. Conazoles were selected in conjunction with the developmental studies planned for program project 3.
- In collaboration with other ORD Laboratories/Centers, develop exposure models.

Project 2 - Determine Age-Related PD Changes. The objective of project 2 is to delineate the magnitude of age-related differences in sensitivity to pesticides and elucidate mechanisms (kinetic and/or dynamic) underlying this differential sensitivity. The approach has involved systematic comparisons of the dose-response and time-course of pesticide effects in rats of different ages, followed by development and testing of hypotheses concerning the mechanisms for these differences. Behavioral and neurochemical (cholinesterase [ChE] inhibition) evaluations have been used to characterize the acute dose-related effects at the time of peak effect of several anticholinesterase pesticides. One outcome of prior research in this area has been to emphasize the effect of kinetic differences in detoxification pathways and to suggest that the age-related differences in kinetics can predict overt toxicity. Research will continue in this area to characterize the absorption, distribution, metabolism, and excretion of

ChE-inhibiting pesticides in young and adult animal models.

Project 3 - Evaluate Developmental PK of a Class of Prototypic Pesticides. This project will evaluate developmental PK information on selected conazoles associated with developmental toxicities. It serves as a case study (as described in project 1 above) and is a component of the harmonization proposal addressing modulation of P-450s and other xenobiotic metabolizing enzymes using conazoles as a case study (see Section 4). The PK factors evaluated will be useful both for better understanding extrapolations across age groups and across species. The following studies will be performed.

- Determination of the ability of selected conazoles to transfer to mother's milk in rats, and
- Comparison of tissue dosimetry of selected conazoles in pregnant and non-pregnant females, as well as assessing the tissue dose to the fetus**.**

Impact

This program project will identify approaches to address PK and PD issues related to extrapolation across windows of vulnerability in multiple species. This will provide options for risk assessment or for additional research to improve those risk assessments. This research will also define for developmental endpoints and chemical classes what risk assessment approaches are feasible addressing windows of vulnerability and what additional work is required to reach a systematic set of guidelines for addressing these issues of interspecies extrapolation for exposure and dosimetry. Finally, the development of computer simulation models that can be parameterized for different species and developmental time periods will be useful for future development of guidelines dealing with PK and PD issues across different life stages.

Cross-Agency Interactions

This program project involves major collaborative efforts between NHEERL, NCEA, and NERL arising, in part, from internal funding. The selection of case studies and the implementation of the research is being done jointly.

Program Project 6: Long-Term Effects of the Developmental Environment

Objectives

- Develop animal models to evaluate effects of developmental perturbation on adverse health outcomes during adulthood,
- Assess key health outcomes during adulthood following developmental perturbation,
- Investigate mechanism(s) of adverse health effects in adults following developmental perturbation, and
- Provide input into interpretation of low birth weight data from Agency testing guideline studies.

Scientific Approach

Clinical and epidemiological studies have shown significant correlations between conditions during development and various diseases later in life. In humans, low birth weight has been used as a surrogate for adverse developmental conditions, but the specific conditions affecting birth weight are usually unknown. Human studies demonstrate inverse correlations between birth weight and risk of later disease, and these correlations hold even within the range of normal birth weight. Adult-onset diseases with which birth weight has been inversely correlated include hypertension, coronary heart disease, diabetes, obesity, schizophrenia, and early onset chronic renal failure (Godfrey and Barker, 2001). There is also evidence that, for female fetuses, the *in utero* environment can significantly affect pregnancy outcome in adulthood. Studies have shown that reduced infant birth weight and other adverse effects are more closely correlated with the mother's weight at her birth (i.e., maternal intrauterine environment) than with her infant's prenatal environment (Rhind et al., 2001). While birth weight has been used as a convenient and available marker of developmental conditions in humans, it is important to make clear that adverse developmental conditions do not always affect birth weight although such adverse conditions may still have long term effects.

The most common adverse effect seen in standard rodent developmental toxicology studies is reduced fetal weight at term. Current Agency guidelines for interpretation of developmental toxicity studies state that fetal weight is an adverse outcome on par with death and malformation, the other adverse outcomes detectable in fetuses at term. However, the interpretation of reduced fetal weight is a contentious subject, especially when fetal weight is affected only at maternally toxic exposures. Current study designs do not examine the long term health of offspring exposed during development (*in utero* and/or postnatally). The utilization of animal models will help to define the causal factors and identify long-term effects under controlled experimental conditions that are impossible to duplicate in epidemiological studies. Current developmental toxicity tests do not address this important issue and may be missing important adverse effects highly relevant to the human condition. Maternal undernutrition during pregnancy in rats has been shown to cause hypertension, diabetes, and other metabolic derangements, as well as adverse neurological sequelae in offspring. This program project will examine the long term health effects of toxic exposures during development. The overall goal of this program project is to determine the conditions under which developmental perturbations will lead to adverse health outcomes at adulthood.

There is evidence that some environmental contaminants may alter developmental

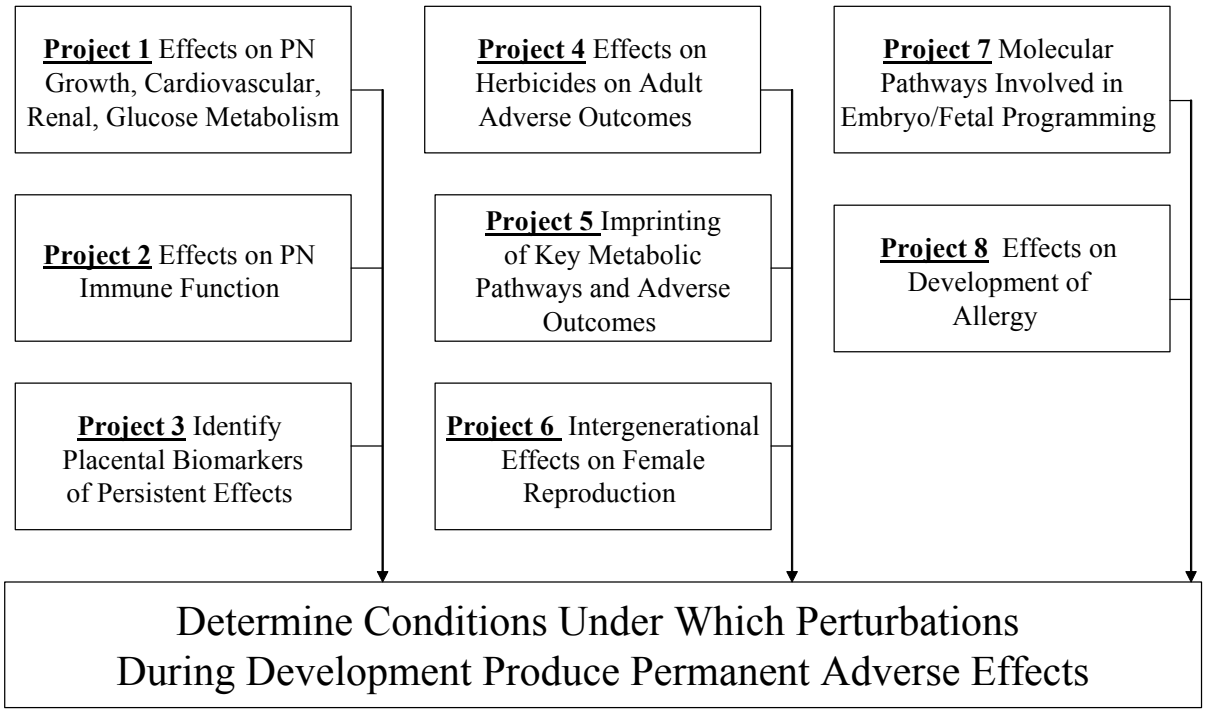

Figure 11 Critical Milestones for Research on Developmental Environment

programming in a manner that does not necessarily result in malformations but affects function later in life. The best-studied examples of this are environmental agents with endocrine activity, and these agents typically affect the reproductive tract or reproductive function. Effects of these agents may not become apparent until puberty, or in some cases, may only hasten the onset of reproductive senescence much later in life. Latent health effects due to developmental exposures to other classes of agents have received scant attention, but there are examples of neurological and neurodegenerative effects of developmental exposures. There is evidence that *in utero* exposure to PCBs leads to altered thyroid function and learning disabilities later in life. The mechanisms underlying these effects are poorly understood, but well-designed studies and new technologies such as gene expression profiling should begin to reveal clues to the developmental pathways involved. Studies using this technology will be designed and incorporated into this program project as results emerge from the more descriptive animal studies. Critical milestones associated with this program project are summarized in Figure 11.

Project 1 - Effects of Developmental Toxicant Exposure or Undernutrition on Adult Physiology. Significant decrements in fetal weight are the most common adverse outcome of rodent developmental toxicity bioassays in which pregnant animals are exposed to xenobiotics. Maternal undernutrition has been studied extensively in rodents and is an established method of inducing lower birth weight in offspring without exposure to toxic chemicals. It was observed over 35 years ago that poor nutrition during pregnancy led irreversibly to reduced cell number in many tissues. It has since been established that maternal protein restriction affects islet cells and insulin-sensitive tissues such as the liver, muscle, adipocytes, kidney and brain in the offspring. Maternal undernutrition also programs offspring for hypertension, and this can occur even when

the undernutrition is only transient during early gestation. Hypertension has also been noted as early as 4 weeks of age in rodent models of fetal programming, and this hypertension persists throughout life (Langley-Evans et al., 1998). Among major organ systems, the kidney appears to be targeted specifically by maternal undernutrition, leading to decreased kidney-to-body weight ratio and fewer nephrons in the kidney at birth (Merlet-Benichou et al., 1994). Prenatal malnutrition resulting in low birth weight pups has also been associated with behavioral abnormalities that persist into adulthood. Cognitive deficits have been reported in a variety of animal models using both behavioral assessments of learning and memory and neurophysiological indices of synaptic plasticity (Kehoe et al., 2001). The hippocampus subserves memory function in humans, nonhuman primates, and rodents and is also a target for glucocorticoids. Neonatal stress induced by isolation induces changes in transmitter function and neuroendocrine activation and contribute to abnormal behavioral and neurochemical responsiveness later in life. Studies in project 1 will expose pregnant rats to toxicants or undernutrition during various developmental windows and offspring will be observed during their entire life span. This research will:

- Demonstrate that physiologic and metabolic development of an organism is shaped in part by its developmental environment, and adverse developmental conditions can produce permanent and deleterious physiological changes which may become apparent later in life.

Project 2- Long-Term Immune Function Deficits Following Immune System Perturbation During Development. Perturbation of immune system development by various stressors has been documented to result in functional decrements that may possibly persist for life. For example, perinatal and/or early postnatal exposure to chemicals such as dioxin, organotins, and organochlorine pesticides has been demonstrated to suppress immune system function later in life (Smialowicz et al., 2001). These results indicate that the developing immune system is more susceptible to perturbation by chemicals and pesticides than is the adult immune system and that these alterations may persist into adulthood, and possibly for life. Research in project 2 will further investigate the conditions under which *in utero* and/or early postnatal stressors lead to permanent and deleterious alterations of the immune system at any stage of post-weaning life. This research will:

- Demonstrate that results of standard immunotoxicological assays (e.g., antibody responses, T cell-mediated responses, cytokine protein and message analysis, and flow cytometric phenotype analysis of lymphocyte subsets) in the rat are useful predictors of long-term changes in immunocompetence that may occur in humans.

Project 3 - Placental Tissue as a Potential Source of Biomarkers Associated with Intrauterine Growth Retardation (IUGR) and Long-term Health Effects. There is substantial evidence in the scientific literature linking exposure to air pollution, cigarette smoke and other agents to adverse pregnancy outcomes including low birth weight, intrauterine growth retardation (IUGR), eclampsia, hypertension, premature delivery, and effects on maternal health (Schardein, 2000). Many of these responses are associated with physiological and morphological changes in the placenta that affect function, e.g., delivery of nutrients and oxygen to the conceptus. Thus, the placenta can be a source of biomarkers that relate to exposure as well as to health effects. For example, changes in gene expression, enzyme induction/activity, and vascularization, among

other endpoints, can be detected/monitored in placental tissues from at-risk pregnancies and compared with normal term placenta. Project 3 will develop a rodent model to evaluate the effects of low birth weight on future health and responses to toxicants. This research will evaluate the role of altered placental morphology, function, and gene expression in the production of intrauterine growth deficits and the long term health effects associated with IUGR. This research will:

- Provide a basis for comparing animal and human responses to toxicants for the potential to impact intrauterine growth and development.

This project will also complement the research proposed under program project 4 "Identifying and Validating Biologic Indicators of Susceptibility and Sensitivity among Children to Assess Potential Risk of Adverse Health Outcomes Associated with Environmental Exposures." In that program project, human placental specimens will be obtained and examined with genomic, enzymatic, protein expression assays, and proteomic analyses. The identification of biomarkers that occur in both species would facilitate laboratory study of toxicants implicated in IUGR and having long-term health consequences. Research in project 3 will:

- Demonstrate that exposure to toxicants affects growth and function of the placenta, contributing to adverse effects during gestation that persist throughout life and can predispose to disease and disability, and
- Compare human and rodent biomarkers associated with IUGR and adverse health outcomes**.**

Project 4 - Effects of High use Herbicides on Breast and Prostate Development and Life-Time Disease Risk in Humans and Rodents Following Gestational or Lactational Exposures. The overall objective of this project is to determine the effects of gestational exposure to chlorophenoxy and chlorotriazine herbicides on breast and prostate development and to compare their adverse health risk outcomes in the rodent and human. Historically, 2,4-dichlorophenoxyacetic acid (2,4-D) and atrazine have been the top two herbicides used in US agriculture by acre. Although the two families of compounds differ in potential MOAs and metabolic clearance, they have both been shown to have adverse health outcomes following fetal or neonatal animal exposures and little effect in adult animals. These studies will:

- Compare post-exposure health outcomes in animal models with epidemiological data in high chlorophenoxy and chlorotriazine herbicide use counties within the US**.**

Previous studies have shown that late gestation exposure to atrazine caused impaired mammary gland development in dams and their female offspring, increased incidence of prostatitis and lipomas in male offspring (Fenton et al., 2002), and left the female offspring with enhanced sensitivity to the effects of a chemical carcinogen, dimethylbenz[a]anthracene. Another study found that atrazine, via modulation of prolactin levels during early postnatal life, increased the incidence and severity of prostatitis in male offspring. Atrazine and its individual metabolites induced adverse reproductive tissue effects if delivered around the time of puberty. However, there is little information concerning the effects of 2,4-D. Studies in project 4 will:

- Provide data on breast and prostate developmental outcomes and cancer and non-cancer endpoints following exposure to chlorotriazine and chlorophenoxy herbicides during periods critical to target tissue or brain development,
- Provide dose-response information for atrazine and its metabolite mixtures and chlorophenoxy herbicide exposures during pregnancy and lactation in rats, and
- Characterize adult risk to these compounds following childhood exposures in rodent models and in humans**.**

Project 5 - Long-Term Metabolic System Perturbations after Developmental Exposures. Drug metabolizing systems are known to be present to varying extent in tissues of the embryo and fetus and can influence the response of the conceptus to toxic chemicals (Miller et al., 1996). Less understood is how the ontogenetic profiles of drug metabolizing enzymes are influenced by exposure to toxicants and how toxicant exposures during ontogeny of drug metabolizing systems might affect their expression and inducibility during adulthood. Permanent alterations in enzyme expression and/or inducibility due to the developmental environment could be a basis for changes in susceptibility to toxic exposures in later life. Alterations in some of these metabolizing systems could also adversely affect metabolism of endogenous substrates including steroid synthesis. Research in project 5 will:

- Demonstrate that during pre- and postnatal development there are critical metabolic pathways which may be imprinted by the developmental environment, leading to permanent changes in their expression and/or inducibility.

Project 6 - Intergenerational Effects on Female Reproduction. There is evidence that, for female fetuses, the *in utero* environment can significantly affect pregnancy outcome later in life. Studies have shown that reduced infant birth weight and other adverse effects are more closely correlated with the mother's weight at birth (i.e., maternal intrauterine environment) than with her infant's prenatal environment. There is a body of epidemiological data that indicates a correlation between the perinatal environment of a woman and her chances of having an adverse pregnancy outcome. There is concern about the relationship of the health of mothers and the health of infants. Research suggests that the incidence of abnormal pregnancy outcomes including stillbirths in women within lower social classes might be associated with childhood stunting brought on by a poor environment. It has also been shown that the incidence of neural tube defects, stillbirths, and low weight babies is high in women born during periods of general malnutrition. Research in this project will:

- Examine the effects of developmental exposures on adult female fertility, pregnancy maintenance and response to developmental toxicants and
- Determine whether a female's *in utero* environment affects her ability to maintain a normal pregnancy and delivery in adulthood.

Project 7 - Gene-Expression Profiling for the Elucidation of Developmental Pathways Involved in Embryo/Fetal Programming. New technologies for rapidly screening for changes in gene expression offer exciting new avenues for the elucidation of pathways of developmental toxicity. There are a limited number of conserved signaling pathways involved in the process of development, and the functions of these pathways are becoming better understood. Because the biology underlying embryo/fetal programming is

poorly understood, gene expression profiling may be the best approach for beginning to understand the process of embryo/fetal programming. Gene expression microarrays have already been used to examine mechanisms of teratogenesis, but there are no established approaches for carrying out such work. These studies will be initiated only after adverse effects have been established in previous projects. Gene expression profiles will be assessed using microarray technology and real-time quantitative polymerase chain reaction (PCR). The specific developmental times and tissues/structures to be studied will depend on the nature of the effects observed in the descriptive studies. This research will:

- Demonstrate that embryo/fetal programming involves changes in gene expression leading to permanent alterations in postnatal and adult physiology, and these changes in gene expression can be detected at early stages using expression microarray technology.

Project 8 - The Effects of Low Birth Weight on the Development of Allergy. Research in this project will determine if low birth weight resulting from either maternal undernutrition or *in utero* toxic exposure plays a role in allergy development. Respiratory and oral (food) allergies are thought to develop in early life in a genetically predisposed population. This project will:

- Determine if low birth weight affects allergy development by increasing susceptibility to sensitization, and/or by enhancing the severity of allergic responses.

Impact

This project has the potential to change the basic study designs for assessing developmental toxicity and to reveal latent toxic effects not seen before. If the results of this study demonstrate long-term morbidity associated with prenatal and/or postnatal exposures, it may be necessary to extend existing study designs or create new designs to test for such effects. Perhaps some types of morbidity associated with developmental exposures are general in nature, while others are chemical-specific. We may find that no increase in disease is associated with exposures that also affect fetal or early postnatal growth, in which case we may not need additional tests; however, such results would clearly raise the significance of affects on fetal weight or postnatal growth seen using current study designs. These studies thus have the potential to bring significant improvements to the risk assessment process.

Cross-Agency Interactions

This project is being carried out in collaboration with Dr. C. Kimmel of NCEA, who will provide a critical risk assessment perspective to the design, interpretation, and application of these studies.

Program Project 7: Susceptible Populations: Susceptibility Associated with Older Adults

Objectives

- Determine the characteristics that define older adults as a susceptible subpopulation,
- Determine PK and PD basis for differential responses of older adults, and
- Determine how susceptibility in older adults varies across systems/tissues.

Scientific Approach

By the year 2030, one in every five Americans will be older than 65 years, nearly double the current population. Given the burgeoning of this subpopulation, it is critical for planning for the care of the older adults to determine whether environmental exposure to toxic agents can result in an age-related accelerated decline in function or increase in diseases of aging such as cancer. Current testing guidelines do not include toxicological assessments focused on older adults. Research on aged human and animal models will improve the scientific underpinning of risk assessment decisions for the aged as a susceptible population by moving risk assessment toward more biologically-based decisions about uncertainty factors.

The goal of this project is to understand whether responses to environmental insults differ in the aged compared to the young adult population. Research designed to reach this goal will generate data that will identify and prioritize those functions and mechanisms that most lead to age-related decline. By doing so, these studies will address the uncertainty of whether toxicity data collected in young adults provides a sufficient margin of safety to protect against effects that may result from toxic exposure to an aged individual. Older adults comprise a subpopulation that may have special susceptibilities to toxicant-induced dysfunction or degeneration due to the critical characteristics of their life-stage. Understanding the biological basis for a differential response in older adults can provide the rationale for decisions on how to appropriately incorporate the differential sensitivity of older adults into an environmental risk assessment framework.

The candidate mechanisms driving age-related susceptibility that will be considered include changes in PK, age-related alterations in cell signaling, deposition and accumulation of harmful metabolic by-products, natural decline in reserve capacity, cumulative gene replication error-induced alterations, changes in processes specific to the cell cycle, DNA repair, cellular homeostasis, and response to oxidative stress.

This program project includes research in two of the major areas of concern to older adults, i.e. cancer and degeneration in the central nervous system (CNS). The project on carcinogenesis will bring to bear the many aspects of initiation, promotion, repair processes, and homeostasis on the issue of the interaction between aging and toxicant-exposure leading to tumor production. The studies on neurodegeneration concentrate on age- and toxicant-induced changes in response and repair mechanisms and function in the CNS and visual system. Work on response and repair mechanisms in the CNS will be compared to similar work in other tissue systems in the cancer project to see if age-related changes in repair and homeostatic mechanisms are similar in these different tissue types. This will allow us to prioritize particular processes in specific systems. Figure 12 illustrates the critical path for this program project.

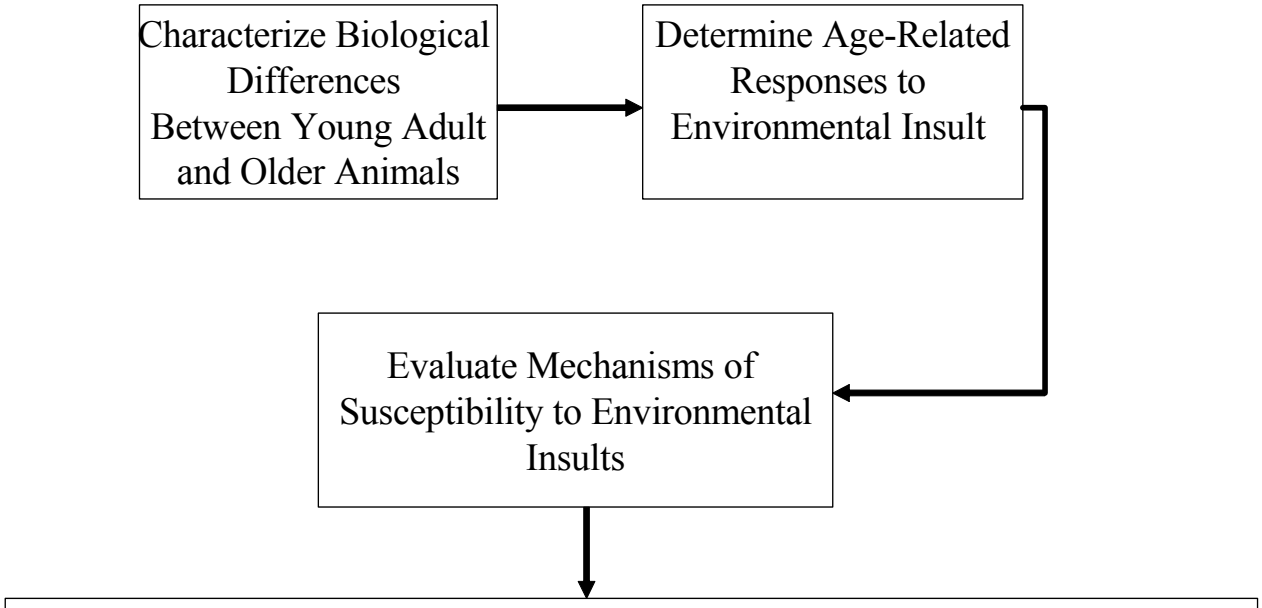

Figure 12 Critical Path for Research on Susceptibility Associated with Older Adults

Project 1 - Mechanisms of Susceptibility to Loss of Neural Function in Older Adults. This project is designed to investigate age-related changes in the CNS and sensory systems at multiple levels. Studies on response and repair mechanisms in the CNS will focus on differences in age- and toxicant-induced gene and protein expression in different brain regions to begin to understand the different processes that are associated with functional losses in these brain areas. The ultimate goal of these studies is to be able to construct a model of the chain of events leading from alterations at the molecular level to alterations at the functional level. To address the broad range of age-related alterations that adversely influence the ability to respond to toxic insult, we propose to measure changes in gene and protein expression for protective, repair, and plasticity mechanisms in the brain as a function of the interactions of age and toxic exposure. As a focused example of the work linking changes at the molecular and cellular levels to changes in functional and morphological endpoints, studies of age and toxicant-induced effects on the retinal degeneration and visual function will be examined. The studies on ocular toxicity address the concern that pesticides may be an environmental risk factor, particularly to aging populations already susceptible to retinal degeneration.

Normal aging processes result in changes in physiological systems that could contribute to increased susceptibility to neurotoxic exposures in older adults. The studies of gene and protein expression will focus on the critical pathways involved in toxic response and detoxification/repair pathways. That is, pathways involved in ROS generation and elimination, proteins involved in maintenance and repair, and genes that have wide ranging function in regulation of cell processes such as transcription factors or homeobox genes and signal transduction pathways. Monitoring global patterns of gene expression allows one to examine multiple physiological

mechanisms simultaneously, gain insight into interactions between mechanisms, prioritize them in order of importance, and to associate them with functional alterations. Because the CNS shares with the other organs an overall decline in cellular and genetic repair and homeostatic processes with aging, we hope to determine if mechanisms that are affected by aging in the brain and nervous system play a similar role in age-induced susceptibility in other systems and for other endpoints (e.g., cancer, non-cancer).

A second set of studies, incorporating some facets of the gene and protein expression work described above, will focus on effects in the visual system in an aging model. The aging visual system is susceptible to ocular toxicity because of a number of factors. Anatomical examination of retinas taken from individuals free of ocular disease show an age-related loss of rod photoreceptors and ganglion cells. Along with these losses is a lifelong accumulation of non-degradable metabolic by-products in and under the retinal pigmented epithelium (RPE). In addition, older individuals represent a growing population uniquely susceptible to loss of vision due to age-related macular degeneration (AMD). AMD is the leading cause of vision loss in individuals older than 65 years; the prevalence of AMD is estimated to increase from approximately 1.6% before age 64 to 28% eleven or more years later. The pathogenesis of AMD is still largely unknown, and it is not clear whether environmental factors may push an individual from the already compromised state due to aging to a state of overt pathology. Broadly-defined chemical exposure, for example, has been identified as a risk factor in AMD. Retinal degeneration has been reported in humans exposed to OP chemicals and in animal models of pesticide exposure and has been linked to both aging and fungicide exposure in epidemiological studies. In addition, there is growing evidence that inflammatory processes play a role in the pathogenesis of age-related macular degeneration and other important diseases of older adults. The proposed studies examine retinal physiology and a second messenger (signal transduction) pathway in the retina known to be affected by OPs in an older animal model, the interactions of light and environmental toxicants in models of retinal degeneration, and a human population exposed to pesticides and showing signs of retinal degeneration. Increased age is a strong co-factor. Research in project 1 will:

- Assess qualitative and quantitative differences in response to toxicant exposure between young and the aged in tissue culture, animal, and human models for neurodegenerative, protective or repair and plasticity mechanisms at functional, cellular, and molecular levels. This work aims to identify mechanisms of susceptibility, targeted sites of neurotoxicant action, and the resiliency of the CNS;
- Correlate age-related alterations with functional endpoints, including brain morphometrics / pathology, function, and behavior;
- Evaluate whether effects observed involve changes in the PK of the chemicals studied; and
- Compare cell and molecular differences found with those determined in other tissues or resulting in other endpoints (e.g., cancer versus non-cancer).

Project 2 - Mechanisms of Susceptibility to Cancer in Older Adults. This project is designed to investigate age-related changes in the body that lead to increased occurrence of cancer. Age is the strongest factor associated with the incidence of cancer in humans. While the delay between an environmental insult and the appearance of neoplastic growth may explain some of this circumstance, research is needed to determine what makes

older individuals preferentially susceptible to the occurrence of cancer. It is possible that environmental agents have greater effect in older individuals. There is general agreement that cumulative damage caused by oxidative stress is the fundamental cause of aging. Additional changes occur in the body when hormone production and maintenance are altered beginning at 40 to 50 years of age. Both of these processes, age-related changes in response to oxidative stress and changes in hormone levels, will be studied in this project because they may be critical to the enhanced cancer susceptibility of the older individual.

Factors mediating increased susceptibility may be present at all stages of carcinogenesis. The older population may be more susceptible to cancer initiation because of age-related alterations in DNA damage, detection and repair–fundamental processes potentially altered by some environmental agents. Damage in or inappropriate expression of specific genes controlling cell proliferation or other functions critical to maintenance of cell homeostasis can lead to uncontrolled cell growth resulting in cancer. Cancer promotion processes may also be affected. These include processes associated with chromosome stability, DNA methylation, suppressor gene activity, signal transduction processes and spindle assembly, all components in the web of promotion processes that can lead to reduced growth control in cells of various tissues. Disruption of a homeostatic process, gap junction communication (GJC), which exhibits tumor suppressor activity, can also lead to increased cell proliferation and cancer formation.

Activities of some hormones are also known to influence the occurrence of cancer. It is possible that changing hormonal levels in older individuals affect the mechanisms of cancer initiation and promotion by environmental agents. For example, the serum levels of melatonin, a known oncostatic agent, decrease as a function of age in humans beginning at 40 to 50 years of age. GJC will be one process studied because it has been shown to be augmented by physiological concentrations of melatonin. In addition, the occurrence of DNA damage will be studied because melatonin has been shown to be one of the most potent free radical scavengers in the body.

Chemicals selected for study will provide experimental validation of principles as well as have direct value in risk assessment analysis. The chemicals include the following: (1) As, which damages DNA, alters GJC, and changes DNA methylation; (2) 2,4-D, which alters GJC and causes liver tumors in rodents; (3) chloral hydrate, which alters GJC but has not been found to cause cancer; (4) potassium bromate and bleomycin, which damage DNA; (5) selected chemicals in the conazole class, which includes pharmaceuticals and pesticides; and (6) dimethyl benzathracene and N-nitroso-N-methylurea, which cause DNA adducts either directly or after metabolic activation, the latter being influenced by melatonin. Ionizing radiation may also be used because it causes DNA damage that can lead to cancer in the absence of metabolic conversion processes, obviating PK concerns. As in project 1 of this program project, much emphasis will be placed on measuring age-related differences in gene/protein expression and chemical toxicity in order to evaluate susceptibilities of older individuals.

These studies will generate directly comparable data across diverse experimental platforms (i.e., whole animal studies, animal cell culture studies, and human cell culture studies). This enhances the value of these studies in that these data may provide critical information needed for extrapolation for the purposes of human health risk assessment. Research in project 2 will:

- Establish animal and cellular models to assess whether older animals are more susceptible than younger adult animals to the carcinogenic effects associated with exposure to specific environmental agents,
- Evaluate the mechanisms of increased susceptibility in older animals, and
- Establish biomarkers of response to estimate risk in humans.

Impact

ORD seeks to provide adequate protection to all segments of the human population, including susceptible subpopulations. In response to a legislative mandate, research has concentrated on effects during development or in the very young. Numerous gaps in the toxicological database still exist, however, for older populations. This program project seeks to examine whether there are special susceptibilities associated with older adults compared to the healthy young adult population. Older adults comprise a subpopulation that may have special susceptibilities to toxicant-induced dysfunction or degeneration due to the critical characteristics of their life-stage. The very young have been recognized as a population of interest in the FQPA and the Agency has recently promulgated an Initiative on Aging that will help guide research in this area in the future. Research on older adults that incorporates age-specific data on biologically effective dose and health effects will improve the scientific underpinning of risk assessment decisions by moving risk assessment toward more biologically-based decisions about uncertainty factors. These studies will address the uncertainty of whether toxicity data provides a sufficient margin of safety to protect against effects that may result from toxic exposure to an aged individual. As such, it should help our understanding of how to appropriately incorporate the differential susceptibility of the aged into the current risk assessment framework of the Agency.

Cross-Agency Interactions

We anticipate interactions with the recently-launched Agency-wide Initiative on Aging, coordinated by Dr. K. Sykes of the Office of Children's Health Protection (OCHP). Research on health effects of environmental exposures to older adults has already been identified in this initiative as one of the critical elements. As this initiative develops, we hope to see opportunities for cross-agency interactions.

Program Project 8: Environmental Risk Factors for Asthma

Objectives

- Identify and rank mold allergens which may be important in the induction of allergic asthma, and
- Assess the effect of air pollutant exposure on the induction and exacerbation phases of the disease.

Scientific Approach

More than 17 million people in the US had asthma in 1998. The incidence of asthma has doubled in the last 20 years; the largest increase has been among children below the age of 15 years of age (CDC, 1998). This increase cannot be explained by changes in diagnostic categorization or by alterations in the gene pool and suggests a strong association between environmental influences and the incidence of disease. About 75% of asthma is associated with allergy, and it appears that the immune system can be programmed to promote asthmatic responses to certain antigens early in life. In nearly 100% of elementary school children with asthma, allergens are the primary trigger for asthma; and their disease is thought to result from early exposure and sensitization to common allergens in their indoor environment (e.g., dust mite, cockroach, molds, animal dander). While some of these allergens have been studied extensively (e.g., dust mite and cockroach), almost none of the mold allergens have been characterized despite their widespread distribution and potential importance in the induction and exacerbation of asthma. A recent National Academy of Sciences Report concluded "There is inadequate or insufficient evidence to determine whether or not there is an association between fungal exposure and the development of asthma" (NAS, 2000).

Epidemiological and clinical studies have also demonstrated that asthmatic responses can be exacerbated by ambient combustion-related products and by domestic and occupational exposures to airborne chemicals. While epidemiological studies have not established a clear role in the induction of asthma for air pollutant or pesticide exposures, animal and clinical studies suggest that ozone, nitrogen dioxide, residual oil fly ash, and diesel exhaust particles can act as adjuvants to enhance allergic sensitization. In addition, recent studies have found that children in inner cities or living closer to major highways have more asthma than children in rural and suburban centers or living further from heavily trafficked roads. Clinical and epidemiological studies have also shown that children with asthma are especially susceptible to the respiratory effects of ambient combustion-related pollutants such as sulfur dioxide and ozone.

The recent Agency's *Asthma Research Strategy* (US EPA, 2002) describes research needs related to susceptibility factors contributing to asthma and risk assessment issues such as dose and type of pollutant which can increase the incidence or enhance severity of allergic asthma. This NHEERL *Human Health Implementation Plan* addresses several classes of environmental pollutants identified by the asthma research strategy including bioaerosols (i.e., endotoxin, molds and other allergens) and combustion-related products formed from the burning of organic fuels. The critical steps for this program project are illustrated in Figure 13.

Project 1 - Fungal Antigens as Initiators of Allergic Asthma. The hypothesis for this research is that the structure and function of the protein constituents of mold isolates determines their allergenicity and can be used

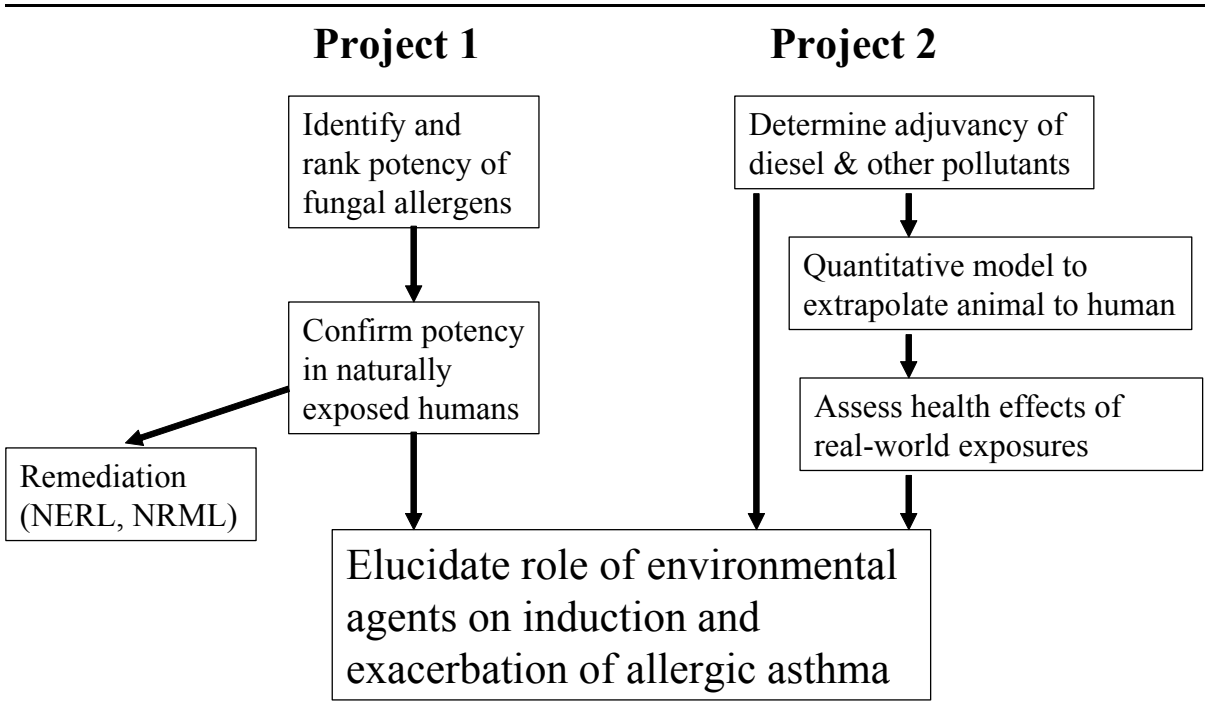

Figure 13 Critical Path for Research on Asthma

to determine the relative potency of different mold components. This project will use a mouse model to develop a cause-effect relationship between exposure to fungal extracts (and specific protein components) and asthmatic responses; characterize the specific protein allergens from three fungi (*Metarhizium anisopliae*, *Stachybotrys chartarum*, and *Penicillium chrysogenum*), look for common structural characteristics, and assess the potency of these allergens relative to known human allergens. Studies in the project will:

- Identify those proteins in *Stachybotrys chartarum*, and *Penicillium chrysogenum* crude extracts that elicit an allergic antibody response (immunoglobulin E, IgE);
- Confirm that the proteins identified as IgE inducers are the relevant allergens in the mouse disease model and determine if these proteins are allergenic in humans;
- Characterize the physico-chemical properties of the allergenic protein(s) from the three fungi and compare to other well-characterized protein allergens such as dust mite allergens; and
- Determine the relative potency of the fungal allergens compared to other allergens such as dust mite, cockroach, and alcalase.

Project 2 - Effect of Xenobiotic Exposure on the Incidence and Severity of Allergic Asthma. The hypothesis for this research is that endpoints reflecting pollutant-enhanced severity of allergic asthma seen in experimental animals can be successfully modeled *in vitro* (cell culture systems) and that quantitative relationships exist between animal and human cell responsiveness. Research in this project will develop laboratory-based assays which can predict the ability of various pollutants to enhance the induction or exacerbate the severity of allergic

asthma, in a dose dependent manner. This approach will be tested using a prototype pollutant (diesel exhaust particles [DEHP]) in *in vitro*, *in vivo* (animal and controlled human studies), and epidemiological studies. The validated paradigm will be used to test the relative potency of a variety of different air pollutants and materials of interest. Research in this project will:

- Compare the genetic expression and release of pro-inflammatory chemokines, receptors, and other immunoregulatory molecules in healthy and allergic airway epithelial cells and macrophages from humans and rodents after incubation with diesel exhaust;
- Correlate *in vitro* findings with *in vivo* markers of disease (altered gene expression and cytokine output, immune function and patho-physiology) in rat and mouse models of allergic lung disease;
- Compare qualitative and quantitative aspects of animal data with clinical results from human studies investigating the effect of DEHP exposure on non-asthmatic and asthmatic lung responses;
- Utilize *in vivo* animal techniques and *in vitro* animal and human assays to screen chemicals and obtain relative potencies for air pollution particles and gases of interest;
- Develop methodology for preparing, storing, and transporting clinical samples for subsequent immunological analysis; and
- Assess the prevalence of biological markers of exposure to DEHP and biological markers of immunological function across intra-urban gradients of combustion-related products and air toxics among school-age children.

Impact

The results from this program project will greatly contribute to the understanding of environmental risk factors for asthma and will address several key Agency needs including species extrapolation and quantitative risk assessment, identification of susceptible subpopulations, aspects of children's health, as well as assessing the health effects of air pollutants (both indoor and ambient) (ORD, 1999). This work will also contribute to the identification of biomarkers of both exposure and effect that will be subsequently used in the NCS (see program project 4 in this Section).

Cross-Agency Interactions

The first project has a strong inter-laboratory collaboration between NHEERL scientists and NERL engineers and biologists in Cincinnati. In the second project, NRMRL engineers are developing a diesel generation and exposure system for use in the *in vivo* studies performed by NHEERL scientists. The work from both these projects will be transmitted to the Office of Air and Radiation, as well as to risk assessors in NCEA.

Program Project 9: Environmental and Genetic Interactions in Hypertensive Rats: Oxidative Stress as a Common Susceptibility Attribute for Non-Cancer Risks

Objectives

- Determine if oxidative stress is a common susceptibility attribute for a variety of environmental insults, including air pollutants, pesticides, and herbicides;
- Evaluate potential mechanisms and biological pathways responsible for susceptibility to toxicants in the presence of preexisting oxidative stress conditions; and
- Determine genetic contribution to oxidative stress and susceptibility to toxicants**.**

Scientific Approach

Individuals with preexisting disease conditions are likely more susceptible to environmental exposures, and often the uncertainty factor employed in risk assessment may not be adequate to protect these individuals. Although diseases such as congestive heart failure, atherosclerosis, Parkinson's and Alzheimer's diseases, diabetes, infertility, and cancer have diverse etiologies, they have one common element, namely, the presence of oxidative stress (Forsberg et al., 2001). Genetic susceptibility to oxidative stress has been proposed in individuals with diseases. Compensatory antioxidant mechanisms are activated in response to injury and the injury in healthy individuals is followed by a normal tissue repair. However, in genetically predisposed individuals with chronic preexisting diseases, these compensatory mechanisms are defective either due to the presence of genetic polymorphisms or a presence of disease; therefore, these individuals are at increased risk of morbidity and mortality from environmental exposures.

One of the significant compensatory antioxidant mechanisms involves regulation of glutathione and a number of phase II metabolism enzymes. Synthesis of glutathione is regulated by γ–glutamyl cysteine synthetase/ligase (γ-GCS). The gene expression is under the control of nuclear factor erythroid-derived 2, like 2, (NRF2) nuclear translocation and binding to antioxidant response element consensus sequence present in phase II metabolism enzymes. This protective transcriptional pathway is induced by a variety of toxicants which result in induction of γ-GCS, glutathione S-transferase A1, heme oxygenase-1 (HO-1), NADPH quinone oxidoreductase-1 (NQO-1), superoxide dismutase-1, thioredoxin, catalase, and uridine diphosphoglycosyl transferase 1a6. Recently, it has been shown that NRF2 knock-out mice showed increased toxicity to a variety of toxicants. It has also been shown that a sensitive (C57BL/6J) mice strain is more susceptible to oxygen-induced pulmonary damage than the C3H/HeJ strain, and that the difference in sensitivity appears to be associated with mutation of NRF2 promoter (T→C, bp-336). This mutation is predicted to add an Sp-1 transcription factor binding site in the C57BL/6J mouse strain compared to C3H/HeJ strain. A recent study also reported a single nucleotide polymorphism at -588 in human γ-GCS which decreases expression of this enzyme and levels of plasma glutathione in myocardial infarction patients. Thus, these mutations are likely candidate single nucleotide polymorphisms associated with susceptibility to oxidative stress in animals and humans.

To understand the mechanism of susceptibility, and the role of underlying genetic oxidative stress as a common susceptibility attribute, relevant animal models exhibiting genetic predisposition to oxidative stress can be employed. This proposal involves the use of genetically predisposed Spontaneously Hypertensive (SH) rats as a model for increased oxidative stress and compare to healthy normotensive Wistar Kyoto (WKY) rats. SH rats are genetically predisposed to increased oxidative stress and have compromised compensatory ability to increase tissue antioxidants in response to mild to moderate tissue damage (expected from environmental exposures). The hypothesis underlying this program project is that a rat model exhibiting phenotypic predisposition to oxidative stress (associated with hypertension/cardiovascular disease [CVD]) will show measurable susceptibility to a variety of insults affecting different organ systems. The research done under this program project will determine susceptibility of SH versus WKY rats to known toxicants that affect pulmonary, cardiovascular, neuronal, ocular, and reproductive systems, as well as investigate the roles for oxidative stress in the exacerbation of the toxic response. This will establish a base for reducing uncertainty factors for individual populations who are at increased risk of environmentally induced disease because of underlying genetic predisposition to oxidative stress.

All individual projects listed below will plan studies in three phases. In the first phase, a dose-response characterization will be done with acute exposure scenarios to determine the level of susceptibility in SH versus WKY rats in several organ systems using appropriate organ specific toxicants. To understand the role of oxidative stress and the mechanism of increased susceptibility, the second series of studies will involve manipulating the oxidative stress of SH rats using antioxidant treatment singly or as a mixture (i.e., tempol, lazaroid, nacysteiline, ascorbate, glutathione). Such agents will be administered prior and/or during their exposure to specific toxicants mentioned under individual projects. The antioxidant regimen that is most successful in reducing systemic oxidative stress will be given consideration for the purpose of this proposal. Antioxidants and biomarkers of oxidative stress will be evaluated in all projects in addition to determining organ specific toxicity markers. Tissues harvested from these studies will also be employed to investigate oxidative stress-related signal transduction (MAP kinases and protein kinase C [PKC]-mediated signaling) and gene expression using comprehensive commercial rat gene arrays. In the third phase of studies, tissues from SH and WKY rats from selected studies will be saved for isolation of DNA in order to screen for possible polymorphism in select gene regions (specifically genes for NRF2, NQO-1, HO-1, and γ-GCS) based on historical evidence of polymorphisms in susceptible humans and animals. These studies will establish the mechanistic basis for genetic susceptibility to oxidative stress, and its implication in risk assessment. The polymorphisms will also be evaluated in patients with CVD and Chronic Obstructive Pulmonary Disease (COPD) which will confirm the role of oxidative stress in human susceptibility and establish a genetic link between animal and human susceptibility traits. The critical milestones for research in this program project are summarized in Figure 14.

Project 1 - Genetic Predisposition to Hypertension-Associated Oxidative Stress and Increased Cardiopulmonary Responsiveness to Ozone and Zinc. This research addresses the hypothesis that SH rats will have exacerbated pulmonary injury and cardiovascular effects from exposure to ozone and zinc. Furthermore, this exacerbated

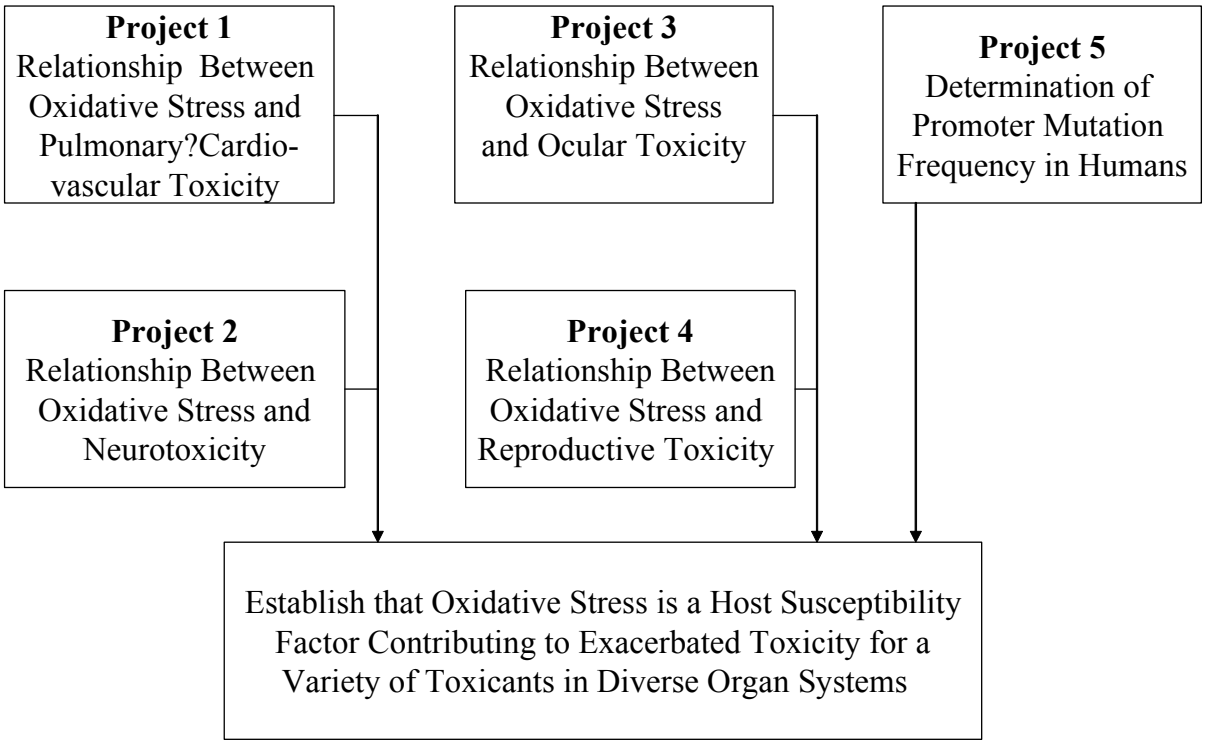

Figure 14 Critical Milestones for Research on Oxidative Stress

response will be diminished in SH rats when pretreated with antioxidants due to diminished oxidant-mediated cell signaling and activation of inflammatory mediators. Ozone has been selected based on its specific oxidant action and its importance as one of the six criteria air pollutants, while zinc is selected based on its likely cardiac effects and its potential role as one of the causative constituent of ambient PM. Research in Project 1 will:

- Determine relative susceptibilities of SH and WKY rats to cardiovascular and pulmonary effects of ozone and zinc sulfate;
- Use antioxidant treatment to establish putative mechanism of action for oxidative stress in cardiovascular and pulmonary effects of air pollutants;
- Assess cardiovascular function using radiotelemetry prior to, during, and after exposure to air pollutants; and
- Determine comprehensive gene expression profile of the lung and the heart and measure oxidative stress cell signaling and specific nuclear factors activation.

Project 2 - Susceptibility of Spontaneously Hypertensive Rats to the Neurotoxic Effects of Pesticides: the Role of Oxidative Stress. Oxidative stress is a condition affecting tissues and cellular functions in a number of disease states. In healthy organisms, ROS which mediate oxidative stress, are generated during normal mitochondrial bioenergetics and are scavenged by various enzymatic and non-enzymatic antioxidants (e.g., glutathione, catalase, xanthine oxidase, ascorbic acid, uric acid). However, if the individual has genetic errors which amplify the formation or reduce the scavenging of ROS, this compensatory response is reduced, resulting in a heightened

state of oxidative stress to cells and tissues. Clinical and experimental evidence indicates that oxidative stress underlies the neurodegenerative condition of Parkinsons Disease. This disease is thought to be a multifactorial condition involving a genetic predisposition to errors in energy metabolism (i.e., mitochondrial complex I), environmental exposure to chemicals that target energy metabolism (e.g., certain pesticides) and a high susceptibility of mesencephalic dopaminergic neurons and glia to oxidative stress and energy depletion. Project 2 will examine the interplay of such factors (i.e., genetics, chemical exposure, neurodegenerative diseases) in SH rats, a strain which has a genetic predisposition to oxidative stress. The first phase of this study will compare behavioral and neurochemical endpoints of oxidative stress in response to ChE-inhibiting pesticides (carbamates, OPs) and paraquat. Endpoints that are representative of oxidative stress will be measured. Brains taken from representative animals showing both behavioral (i.e., reduction in locomotor activity) and neurochemical changes will be examined for neuropathological changes. *In vitro* experiments will examine the effects of complex I inhibitors (e.g., paraquat, rotenone) on mesencephalic glia and neurons. The effects of antioxidant pretreatment on pesticide-induced behavioral and neurochemical changes will then be examined in whole animals, their brain tissues, and CNS target cells *in vitro*. It is hypothesized that because of their condition of chronic stress and reduced ability to scavenge ROS, SH rats will show an increased susceptibility to the neurotoxicity associated with selected pesticides. Research in this project will:

- Compare behavioral, neurochemical, and neuropathological endpoints of oxidative stress in response to various pesticides in animals;
- Describe the morphological and neurochemical response of PD target neurons and microglia to complex I inhibiting pesticides;
- Determine activation of PKC and MAP kinase signaling and gene expression patterns in response to pesticide exposures; and
- Determine the protective effects of anti-oxidant treatment on pesticide-induced neurotoxicity.

Project 3 - Ocular Toxicity and Oxidative Stress. Visible light, the primary stimulus for the visual system, is also its primary toxicant. Many xenobiotic compounds absorb light in the ultraviolet or visible wavelengths and produce ROS (singlet oxygen) in response. This project will investigate whether the mixture of light-induced damage is a mechanism for the generation of ocular toxicity of the retina and the lens. The hypothesis for this project is that xenobiotic chromophores will absorb light and produce damage through oxidative stress in ocular structures and this damage will be exacerbated in animal models deficient in protective mechanisms due to either inherited characteristics, e.g., as in SH rats, or due to dietary deficiencies in antioxidants. Antioxidant supplementation would likely reduce damage. Research in this project will:

- Use physiological, morphological and biomicroscopic endpoints to assay damage to the retina and lens following exposure to insecticides and fungicides; and
- Determine levels of ROS in specific ocular tissue to establish connection between oxidative stress and retinal damage.

Project 4 - Role of Oxidative Stress in the Reproductive Function of Males and Females. Epidemiological studies have shown that certain subpopulations of people have lower blood levels of glutathione and may be more prone to oxidative injury in somatic cells. Research in this project will address the possibility that such individuals

may be at increased risk of adverse reproductive effects associated with the induction of oxidative stress in gonadal tissues. The hypothesis for this research is that SH rats will have a diminished testicular response to oxidative stress as compared to the WKY strain and that lower amounts of glutathione and decreased induction of glutathione synthetic enzymes may be found in the challenged SH animals. Differences in levels of NRF2 would suggest a role for this transcription factor in the altered response. Sperm motility may also be affected in these animals due to increased membrane lipid peroxidation in the presence of diminished glutathione levels. These animals will also serve as positive and negative controls for subsequent experiments (DNA array analysis). The hypothesis for this research is that diminished response to oxidative stress may exacerbate the effects of known testicular toxicants such as acrylamide and thiocarbamate pesticides such as thiram or molinate. Finally, the effects of environmental toxicants on zygote formation in SH and WKY rats will be tested. This research will:

- Determine if the SH rat is significantly more susceptible to toxicant-induced oxidative stress/antioxidant levels than normal rats,
- Determine oxidative stress-induced responsive gene expression pattern in testis following exposure to toxicants in WKY and SH rats; and
- Determine the biomarkers and the role of oxidative stress in susceptibility to toxicants.

Project 5 - Oxidative Stress in CVD and COPD Patients and Possible NRF2 Promoter Mutation. Lower levels of antioxidants and systemic oxidative stress have long been considered risk factors associated with CVD, COPD, and other chronic diseases. The presence of genetic mutations in antioxidant compensatory genes and their role in pathobiologic mechanisms of disease development are not well understood. To determine if NRF2-mediated compensatory antioxidant mechanisms are involved in predisposition to oxidative stress, selected gene mutations (specifically genes for NRF2, NQO-1, HO-1, and γ-GCS) that are known to exist in humans and animals will be evaluated in SH rats and humans with CVD and COPD. This study will allow us to establish the genetic basis to oxidative stress and consideration of genetics in relevancy of animal model to human diseases and will

- Correlate the detection of increased mutation frequency in NRF2 promoter and other related genes with that of the severity of disease in humans and its incidence in SH rats.

Impact

Currently an uncertainty factor of 10 is employed in risk analyses in order to account for differences in susceptibility due to host preexisting conditions and genetics. However, recent epidemiological evidence demonstrates that in many instances susceptibility may lead to increased mortality or disease such that an uncertainty factor of 10 may not be adequate. There is often incomplete knowledge regarding the existence, extent, and characteristics of individual susceptibility about who is susceptible and to what extent. Identifying the mechanisms that underlie susceptibility to many environmental toxicants that play a role in the development or exacerbation of disease leading to death is important in both basic risk assessment and reducing the uncertainty.

Research in this program project will also utilize a relevant animal model to determine increased susceptibility to a variety of toxic insults and organ systems due to underlying oxidative stress/disease. Knowledge of the mode or mechanism of action for specific agents will reduce uncertainties in their risk assessment. Mechanistic work generating in this program project may also lead to the

identification of new biomarkers of toxicity, as well as susceptibility to toxic insult.

This research will also lead to the development and use of technologies to evaluate gene expression profiles to understand patterns of pathological processes and identify potential gene polymorphisms to better define susceptibility. These technologies will lead to a greater understanding of the key events associated with adverse effects. Thus, the data generated from the gene expression profiling studies will be useful to the emerging Computational Toxicology research program.

Cross-Agency Interactions

As results are produced, collaborations with NCEA and Program Offices will be developed.

Program Project 10: Genotype and Phenotype in the Metabolism, Toxicity and Carcinogenicity of Arsenic (As)

Objectives

- Identify genetic polymorphisms for enzymes that metabolize As,
- Examine the regulation of the gene and the role of nutritional status in its regulation,
- Examine the function of arsenate reductase as a critical factor in metabolism of As, and
- Examine As-protein interactions to gain insight into the mode of toxicity for this metalloid.

Scientific Approach

It is known that genetic polymorphisms and mutations can greatly affect human susceptibility to mutation and/or cancer (e.g., fast and slow acetylators of aromatic amines and bladder cancer, xeroderma pigmentosum, Cockayne's syndrome and trichothiodystrophy). Genetic polymorphisms and mutations may significantly affect the outcome of exposures to chemical stressors, such as As. Given the relatively high frequency of polymorphisms in the human population, this proposal will address the potential effect of specific polymorphisms on responses to environmental exposures.

This research will study the role of genetic factors in the expression of toxicity by investigating a prototypic compound having environmental relevance, i.e., As. There is a consensus that understanding variability in the metabolism of iAs in humans is critical to understanding the response of humans to chronic exposure to iAs (Lin et al., 2002; National Research Council, 1999). Inter-individual variation in absolute and relative amounts of methylarsenic and dimethylarsenic in urine has been attributed to differences in ethnicity, age, sex, nutritional status, and extant disease. In humans chronically exposed to iAs in occupational and environmental settings, considerable inter-individual variation has been found in the occurrence of specific signs and symptoms of As intoxication. For example, only a small fraction of individuals chronically exposed to iAs in drinking water develop blackfoot disease, a progressive occlusive disorder of the peripheral vasculature. A role for metabolic capacity in determination of susceptibility is supported by studies in chronically-exposed populations that find absolute or relative increases in the amount of metabolites of As in urine are associated with increased occurrences of As -induced skin lesions.

This program project will determine whether the inter-individual variation in response is related to differences in the capacity to metabolize iAs, if these differences in metabolic capacity are primarily determined by the kinetic properties of the enzymes that catalyze the reduction of pentavalent As and the methylation of arsenicals, if the kinetic properties of these enzymes are determined by their primary sequence (hence, by the genotypes for these proteins), and whether control of the expression of these genes may be an important in determination of the capacity for metabolism of As. As a corollary to these postulates, this research will determine if there is a linkage between the genotypes for these enzymes and the disease susceptibility phenotype which is manifested by the capacity of cells or organisms to metabolize iAs. It is likely that polymorphisms in the genes which encode these proteins determine their catalytic activities and that the transcriptional regulation of these genes affects their expression. If susceptibility to the adverse effects of chronic exposure to iAs is related to capacity to produce methylated metabolites, then genotypes for these critical enzymes are highly relevant to predicting the hazard associated with chronic exposure to this

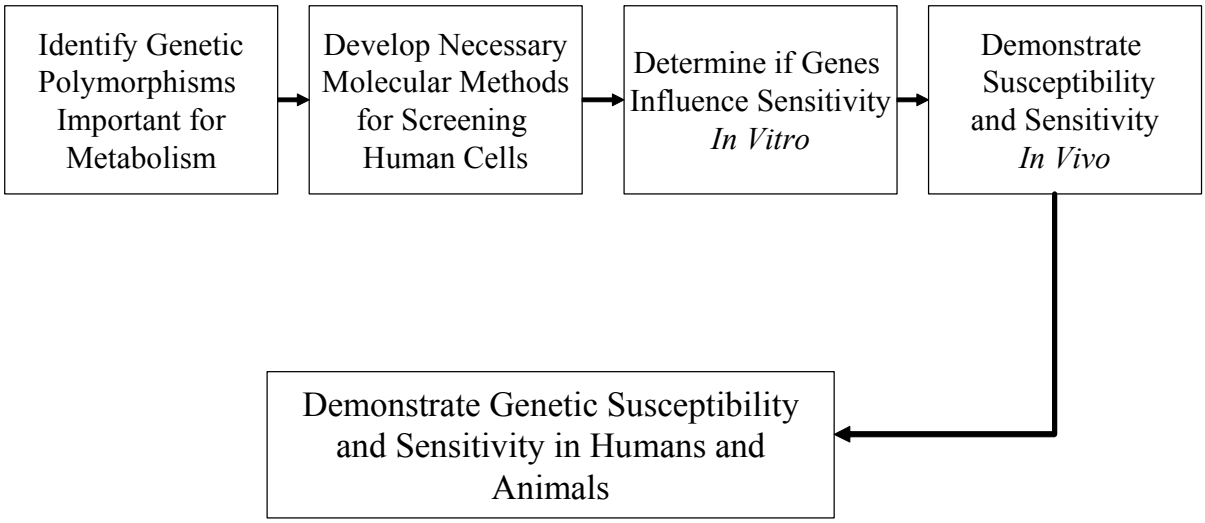

Figure 15 Critical Path for Research on Genetic Polymorphisms

metalloid. Studies of genotypic-phenotypic relations in human cells and in individuals are important steps in estimating the role of interindividual variation in determination of susceptibility. The critical steps for research on genetic polymorphisms are summarized in Figure 15.

Project 1 - Genotype-Phenotype Correlations for As Methyltransferase in Humans. Research in this project will examine the relation between the genotype of Cyt19, a gene encoding the human As methyltransferase and the phenotype for the capacity to methylate iAs in human cells and in individuals chronically exposed to iAs. The hypothesis for the proposed research is that the genotype of Cyt19 affects the phenotype for As metabolism as exemplified by the pattern and amounts of iAs and metabolites produced by cultured human cells or present in blood or excreted in urine by chronically exposed individuals. The specific aims for this study are (a) to examine in cultured primary human hepatocytes, the relation between the Cyt19 genotype and the phenotype for the metabolism of As, as reflected by the amounts of iA and metabolites in cells and media; (b) to examine genotypic variation in Cyt19 using DNA isolated from peripheral blood of a number of human volunteers; and (c) to examine the relation between the Cyt19 genotype and the phenotype for the metabolism of As as reflected by the amounts of iAs and metabolites in blood and urine from humans chronically exposed to iAs in drinking water. Research in project 1 will:

- Examine the relationship between Cyt19 genotype and phenotype in cultured primary human hepatocytes from 20 to 30 donors,
- Examine the genotypes for Cyt19 using DNA from a larger number of human donors; and
- Study genotype and phenotype in individuals who are chronically exposed to iAs.

Project 2 - Genetic Susceptibility and Sensitivity to As Exposures. This project will address the hypothesis that cells, animals, or individuals who might show polymorphisms at the gene for glutathione transferase omega (GST-O) will be differentially susceptible to the genotoxic and hence carcinogenic effects of As. Research in this project will:

- Determine if polymorphism in GST-O exists and if it may be responsible for differential sensitivity to arsenic genotoxicity.

Project 3 - Altered Phenotype-Binding Studies of Arsenicals and Purified Proteins or Synthetic Peptide Amino Acid Sequences of Importance in As Metabolism Reductase and Cyt19) or Mechanism of Action. This research address the hypothesis that there are polymorphisms in As metabolism and mechanism of action (Kitchin, 2001). Genetic susceptibility to arsenicals will be revealed by the binding of arsenicals to certain amino acid sequences of proteins that are important in As metabolism or mechanism(s) of action. If there is a polymorphism in human Cyt 19, there will be an altered amino acid sequence that can be studied via binding experiments. If there is a genetic deletion or substitution of key sulfhydryl(s) required for the binding of the arsenical would be altered. These studies will:

- Evaluate altered binding affinity and/or amount of binding of arsenicals to polymorphic animal or human proteins.

Project 4 - Interactions of Dietary Folate Deficiency and As: Evaluations of Gene Expression Changes in Association with Enhanced DNA Fragility and Possible Alterations in Methylation Patterns. As toxicity may be enhanced in individuals with folic acid (folate) deficiency. As this vitamin is needed for normal DNA synthesis and methylation, its deficiency can result in effects which interact with those of As via different pathways. Folate deficiency can result in uracil misincorporation and increased DNA fragility, an effect which could be increased by As inhibition of repair enzymes. This deficiency may also lead to a depletion of S-adenosylmethionine required for methylation to result in DNA hypomethylation and possibly decreased As detoxification. These studies will:

- Use a mouse model to evaluate global changes in gene expression associated with folate deficiency, As exposure, and most important, folate deficiency plus As exposure.

Project 5 - Transcriptional Control. To study the transcriptional control of Cyt19 (As [III] methyltransferase) and the expression pattern of Cyt 19 in various human tissues. Cyt 19 has been recently identified as the methyltransferase that catalyzes the formation of methylated and dimethylated As. Methylated arsenicals that contain As(III) are more potent than other forms of arsenicals in regards to genotoxicity (Mass et al., 2001) and inhibition of various enzyme activities. Our hypothesis is that the polymorphisms in the transcriptional activity of Cyt 19 contributes to human susceptibility to mutation and/or cancer caused by As. By understanding the transcriptional control of this gene, we are in better position to understand the human susceptibility to As. Methylation of As is a critical feature of its metabolism, therefore, characterization of Cyt 19 gene and its expression pattern will improve our understanding of the mechanism of As as a toxin and a carcinogen. This research will:

- Delineate the regions and factors that control the expression of the Cyt 19 gene,
- Study the tissue specificity of this gene by looking at the presence or absence of the expression factors in different tissues, and
- Study the expression of this gene in different populations.

Cross-Agency Interactions

We have ongoing collaborations with NERL-Cincinnati to develop and validate new analytical methods that are used in our work on genetic polymorphisms of As metabolism. We also have a ongoing collaboration with Region 10 to examine metabolism of As in individuals ingesting a variety of marine

organisms. This work may include studies of genetic polymorphisms of As metabolism.

Impact

Although most attention has focused on the potential health effects of chronic ingestion of As-contaminated drinking water, substantial exposure (and risk) occurs from other sources. As is a component of particulate material generated by fuel combustion, is introduced into the environment by the use of As-containing pesticides, and occurs in complex organic species (e.g., arsenobetaine, arsenosugars) in many foods. Hence, data from the proposed research on the control of the metabolism and toxicity of As will contribute to risk assessments for chronic exposure to As from water, air, and food. Because exposure to As occurs from each of these media, the availability of new data on the metabolism and effects of As will improve the risk assessment for this element and assist the Agency in its mission to protect public health.

In general terms, the proposed research will emphasize three distinct, but related, aspects of the health effects of As. Understanding the role of genetic susceptibility in the control of As metabolism will benefit the risk assessment process by identifying subpopulations of individuals who are genetically predisposed to greater susceptibility to the toxic or carcinogenic effects of chronic As exposure. Second, experimental evidence may be obtained that show unusual sensitivity to As. Third, the factors that are identified in the proposed research as modifiers of the metabolism and toxicity of As can be incorporated into quantitative models which describe the systemic and cellular metabolism of As and its MOA as a toxin and carcinogen.

This approach would extend current modeling efforts that examine the fate of other forms of As (i.e., total As or total methylated As) and improve the utility of these models in risk assessment. Taken together, the proposed research effort examining the genotypic and phenotypic control of metabolism, toxicity, and carcinogenicity of As will contribute to the Agency's periodic reevaluation of risk assessments for this element in water, air, pesticides, and other media.

4.5 Gap Analysis and Links to Other Multi-Year Plans

NHEERL's implementation plan with regard to susceptible subpopulations focuses on life stage, genetic polymorphisms, and asthma. NHEERL research on children is consistent with research needs outlined the Agency's Strategy for Research on Environmental Risks to Children (US EPA, 2000). NHEERL research on older adults responds to the Administrator's interest and concern about the environmental health of older Americans. Research described in the program project on Aging has been included in the recent inventory of Agency intramural and extramural research projects organized by the OCHP. Research related to older adults is envisioned to increase over the next few years.

NHEERL research on genetic polymorphisms deals primarily with a limited number of possible genes associated with differential sensitivity to the prototypic chemical used in these studies, i.e., As. There is considerable human and animal data available on As, and it is clear that there is a subpopulation of people that respond differently to this metal. As indicated in the program project on genotype and phenotype in the metabolism, toxicity, and carcinogenicity of As, there are several testable hypotheses concerning potential gene-chemical interaction that should provide basic information needed to develop strategies for addressing this issue with other environmental agents from other chemical classes. Research on a non-cancer class of chemicals, such as the CHE-inhibiting pesticides, would provide useful complementary information with regard to gene/chemical interactions. Results

from a cancer and non-cancer agent could be used to help develop risk assessment strategies that include potential gene/chemical interactions in susceptible subpopulations.

NHEERL research with regard to preexisting disease focuses on asthma and is consistent with research priorities outlined in the *Asthma Research Strategy* (US EPA, 2002). Other diseases such as autoimmune deficiency diseases, cardiopulmonary diseases, diabetes, and neurodegenerative disorders such as Parkins disease could potentially interact adversely with exposure to environmental pollutants. NHEERL does not plan to conduct extensive research on these diseases at the present time, although compelling epidemiological research suggesting an interaction between a chemical class and a disease could generate hypotheses that could be tested in animal models.

Research on susceptible subpopulations is also linked to several other MYPs. In the case of Drinking Water, NHEERL research will contribute to other studies to identify the risk of birth defects due to drinking water contaminants, provide fundamental information on the basis of inter-individual variation in As metabolism. For Air Toxics and PM, NHEERL research will help identify the role of specific polymorphisms in the metabolism and repair genes and the relative increase or decrease in tumorigenic risk associated with exposure to air pollutants, identify parameters for models describing the deposition of fine and coarse mode particles in older adults and moderate asthmatics, provide basic research on the effects of concentrated air particles in asthmatic humans and animals, provide data for a report on the acute respiratory effects of PM and co-pollutants in asthmatic children, help describe the effects of metals in animal model of allergic asthma, and provide basic data to help describe the PK of oxygenate mixes in young and older adult populations. For EDCs, NHEERL research on susceptible subpopulations will contribute to the evaluation of toxicant-induced alterations in mammalian reproductive development as a function of critical window of exposure, provide basis information for research on the influence of life stage on the sensitivity and reproducibility of estrogen cDNA macroarrays, help evaluate critical windows of exposure for alterations in mammary gland development and lactational function, and characterize the effects of atrazine delivered during critical windows of development.

4.6 References

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Section 5
Cumulative Risk

5.1 Problem

Cumulative risk is of interest to a wide variety of Agency's Program Offices. For example, the FQPA directs the OPP to include in its assessments of pesticide safety the risk(s) associated with the cumulative effects of chemicals that have a common MOA. The Office of Water (OW) is concerned with risks from mixtures of DBPs and balancing any risks associated with DBPs, as single chemicals or mixtures, against the risks associated with microbial agents in water. Recently, CCL chemicals have become a concern of OW, leading to considerations of cumulative risk for CCL chemicals as well as mixtures containing DBPs and CCLs. The Air Office is concerned with mixtures of criteria air pollutants and volatile organic compounds. The Waste Program is concerned with mixtures of chemicals from the same or different chemical classes that are present in the air, water and soil in and surrounding waste sites. In addition to these chemical-specific concerns, the Agency is concerned with communities that may either have elevated exposure to chemicals or may be subject to stressors such as poverty, lack of access to medical care, or inadequate nutrition.

NHEERL's research will focus on providing data that may be used in development and refinement of risk assessment approaches, particularly addressing principles that may underlie interactions of chemicals at low dose levels (Teuschler et al., 2002). This research will utilize empirical and mechanistic data and models described below, as well as results derived from work on harmonization (see Section 4), to develop strategies to predict the effects of chemical mixtures. Either or both bottom-up (component-based) and top-down (whole mixture) approaches may prove useful for development of mechanistic data and predictive models.

The vast majority of risk assessments for chemical mixtures rely on component-based approaches. This raises a concern with cumulative exposure because exposure to multiple chemicals may interact in ways not predicted or expected based on the dose-response curves of the single chemicals and on an assumption with regard to additivity (i.e., toxicity may be greater or less than expected based on the single chemical information and an assumption of either dose-addition or response-addition). There is also a minor concern with cumulative exposure that toxicity may be seen in an unexpected organ. As dose-additivity for non-cancer health effects is a default assumption on which many Program Offices rely, including OPPTS for OP pesticides, a major goal of this program project is to examine those circumstances under which dose-additivity is a reasonable assumption. Included in this effort is the development of efficient experimental designs and statistical methods for mixtures more complex than 2-3 chemicals.

Another very important research issue is what happens when there are components of a mixture which have different MOAs or multiple mechanisms. As response-addition is the risk assessment default assumption for mixtures of chemicals with diverse MOAs, another major research goal of the program is the examination of the potential for additivity of mixtures of chemicals with different MOAs. Included in this research will be comparisons of the appropriateness and predictive value of response-addition versus dose-addition for various mixtures.

Another important issue for either component-based or whole mixture approaches is whether the quality, e.g., nature (greater than-, less then-, or additive) or

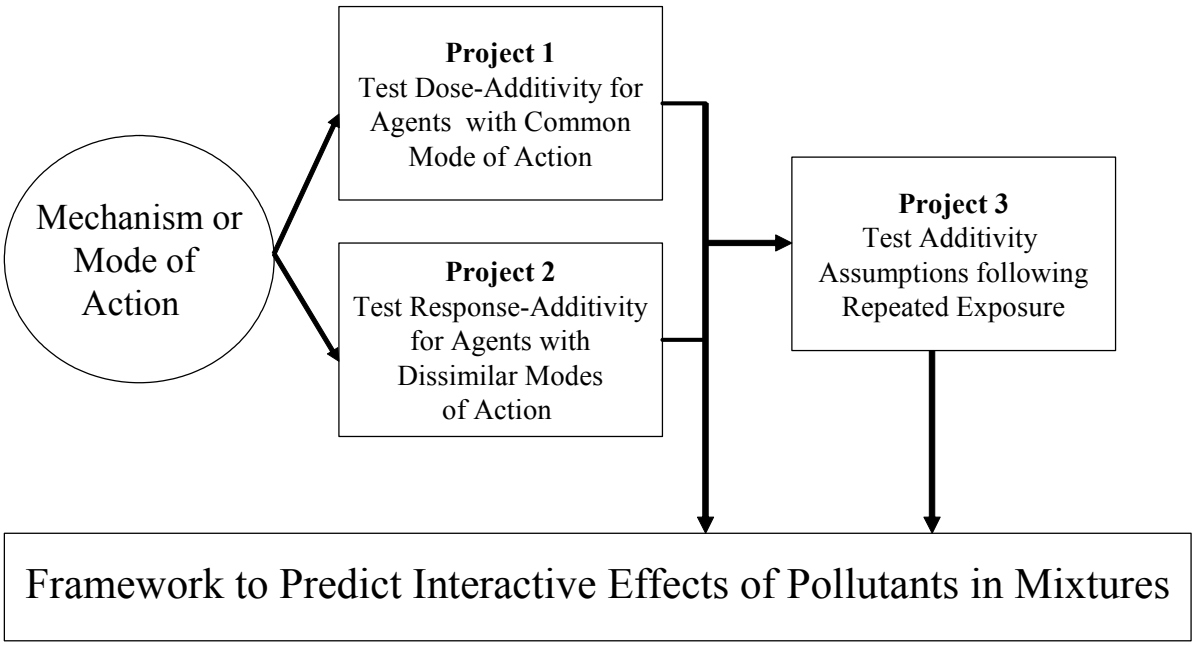

Figure 16 Critical Path for Research on Cumulative Risk

magnitude, of the interactions of chemicals change as the dose level changes (Simmons, 1995) or as a function of repeated exposure. Data are needed to describe the dependence of interaction magnitude on mixing ration, total mixture dose and on component fractions. NHEERL research on cumulative risk will explore the influence of dose and exposure scenario on interactive toxicity. Historically, most mixtures research has focused on short-term exposure to higher portions of the dose-response curve where such situations as saturation kinetics might influence the interactive outcome. In contrast to this historical situation, NHEERL research will include a targeted focus on the low end of the dose-response range and on repeated exposures.

NHEERL research will also address the problem of determining a threshold response for the endpoint(s) of interest at environmentally relevant doses. These may include doses at or below the No-Observed-Effect-Level (NOEL) or No-Observed-Adverse-Effect-Level (NOAEL) of the single chemicals and doses representing either levels found in the environment or at or near the RfD. Doses will be selected to be as close to environmentally relevant levels as permitted by the limits on experimental sensitivity due to the number of animals used. *In vitro* work will make assumptions regarding the extrapolation of experimental doses to the target site concentration *in vivo*. The upper end of the dose-response curves will include effects which are determined to be adverse for the endpoint in question. It should be noted that experiments utilizing the mixture ray design explicitly measure responses over a range of effect levels. Effects will not, however, be characterized at dosages which produce overt toxicity such as animal lethality. The critical path for this research is illustrated in Figure 16.

5.2 Goal

The overarching goal of NHEERL research on cumulative risk is to provide the scientific basis for predicting interactive effects of pollutants in mixtures and the most

appropriate approaches for combining effects and risks from pollutant mixtures.

5.3 Critical Path

Figure 16 outlines the critical steps that will be taken for NHEERL research on cumulative risk. First, either mechanistic data on selected environmental pollutants or assumptions about MOA will be used to design subsequent research on mixtures (see Section 3). A second step will be to study interactions among chemicals with a known or assumed common MOA to determine if the interactions among them are dose-additive. A next step will be to determine if interactions among chemicals with dissimilar MOAs are response-additive. The last step will be to investigate whether the nature (i.e., additive, greater than additive) of the interaction(s) and their magnitude, if non-additive, that result from acute exposure are different from those that result from repeated exposure. Research in this program project will be consistent with APGs and APMs in the Cumulative Risk Section of the ORD Human Health Research Multi-Year Plan (Appendix D).

5.4 Program Project

Only one program project "Exploring the Limits of Additivity" was developed. It is concerned with the assumption of addivity use in risk assessment of chemical mixtures.

Program Project 11: Exploring the Limits of Additivity

Objectives

- Test the assumption of dose-additivity for chemicals with a similar MOAs,
- Test the assumption of response-additivity for chemicals with dissimilar MOAs, and
- Determine if the assumption of dose- and response-additivity changes following repeated exposure.

Scientific Approach

The Agency has published several documents describing methods to perform health risk assessments of chemical mixtures (US EPA, 1986; 1990, 2001) in which three approaches (i.e, actual mixture, similar mixture or component-based) to quantifying health risk for a chemical mixture are recommended depending on the type of data available to the risk assessor. The preferred approach is to utilize data on the complex mixture itself, but such data are often not available. The second approach, which is recommended when data limitations prohibit the use of the first approach, uses data on a "sufficiently similar mixture or a group of similar mixtures." As these data are also very scarce, the overwhelming majority of mixtures risk assessments are conducted with component-based approaches. Thus, this research will focus on component-based approaches to provide information useful to how mixtures risk assessment are actually done in the present and in the future. In addition to this component-based focus, several research projects will take a "mixture as the whole" approach**.**

The overall approach of project 1 of this program project will be to test the assumption of dose-additivity based on the evaluation of several classes of chemicals, including OPs, carbamates, pyrethrins, DBPs, persistent organic pollutants, and azoarene-contaminated water effluent. Project 2 will use the same overall approach to determine if the default assumption of response additivity holds for chemicals having dissimilar MOAs. Project 3 will focus on predicting effects of exposure to selected pesticides following repeated exposure.

Project 1 - Develop and Apply Methods to Examine Whether the Assumption of Dose-Additivity Holds for Mixtures Comprised of Two or More Chemicals Acting via a Common MOA. This project will use relatively low exposure levels for the component chemicals in the mixtures. Many earlier interaction studies have explored the consequences of high dose combinations, i.e., LD_{50}s. Instead, these mixture studies will use low doses, e.g., levels that do not produce overt toxicity and approach more environmentally relevant levels. In addition, attention will be paid to the ratios of chemicals present in the mixture because the proportion of individual chemicals may influence the nature of the interaction.

Research in this project will focus on determining whether an interaction between pesticides in a mixture can be assessed by their actions at specific biomolecular targets, i.e., ion-gated or second messenger-linked neurotransmitter receptors, various enzymes, cellular macromolecules, DNA, proteins, or even specific moieties of a chemical structure. This research will address the hypothesis that compounds that interact with a common biomolecular target (after adjustment for potency and PK differences) will behave in a dose-additive manner. Specific issues to be addressed by these studies include the following:

- Development of molecular and mathematical models for the interactions of pesticides with specific bio-molecular targets,

- Assessment of interactions of pyrethroid pesticides on voltage-gated Na^+ channel function and membrane excitability,
- Assessment of interactions between OP and carbamate pesticides in rats
- Assessment of interactions of mixtures of multiple (3-5) OP pesticides in adult and pre-weanling rats, and
- Assessment of the interaction of multiple carbamate pesticides in adult rats.

Research in this project will also use diagnostic genotoxicity assays to examine low dose additivity assumptions for mixtures of compounds that act via a common MOA. This research will examine the following null hypothesis: regardless of the genotoxic MOAs and the specific type of xenobiotic metabolism, mixtures of genotoxic compounds that act by a common MOA, when applied at low doses, do not depart from the linear dose-additivity assumption. This research will:

- Compare the low dose-response of environmental mixtures and their corresponding "totally artificial mixtures" and the statistical (risk assessment) prediction of the mixture from the artificial mixtures for the environmental mixture;
- Determine if the frequency of "rare spontaneous mutations" for compounds that elicit these rare events provides a means for examining the dose-response curve at extremely low doses;
- Examine the hypothesis that multiple chemicals give an additive response when each chemical is applied at below detectable (response) doses but the modeled additive response gives a minimally detectable response; and
- Address the observation that genotoxic mixtures also have other toxic effects (e.g., immunotoxicity).

Research in this project will develop and use experimental designs and statistical analysis methods for mixtures of multiple chemicals having a common MOA. Chemical mixtures to be evaluated are a chemical mixture comprised of 4 trihalomethanes (chloroform, bromoform, bromodichloromethane and chlorodibromomethane) that are regulated by the Agency; a chemical mixture comprised of 5 HAAs regulated by the Agency (monochloro-, dichloro-, trichloro-, monobromo-, and dibromoacetic acid); and a 9 chemical mixture that contains the sub-mixtures. This research will:

- Extend existing methodology for simple mixtures (Gennings et al., 1997) to mixtures of up to 10-20 chemicals.

Additional research in project 1 will test the interaction of persistent chemicals that are similar in structure and assess their effects on following developmental exposure. This research will explore in *in vitro* and *in vivo* models the effects of chemical mixtures such as the PCBs and polybrominated diphenylethers on key biological events known to be critical for the development of the nervous system, such as PKC. These experiments will test the hypothesis that the interactions of chemicals with a similar MOA follow dose-additivity with regard to PKC and will:

- Develop neuronal cultures (hippocampal and cerebellar granule neurons) to examine the effects of chemical mixtures *in vitro*,
- Construct individual dose-response curves *in vitro* for selected chemicals,
- Determine presence of dose-additivity of binary mixtures, and
- Evaluate dose-additivity of complex mixtures at the low end of the dose-response curve.

Project 2 - Examine the Potential for Response-Additivity of Mixtures of Chemicals with Different MOAs. With few exceptions, the research that has been conducted examining the validity of the assumption of additivity has used mixtures of

chemicals that have either the same MOA or the same target organ. Far less work has examined whether additivity holds for mixtures of chemicals that contain chemicals with different MOAs (diverse mixtures), yet it is to these mixtures that humans are exposed. Current risk assessments assume response-additivity as the default for mixtures of chemicals of dissimilar action. Thus, predictive models that operate in the observable effects region would need to incorporate response-addition for dissimilar chemicals. It is possible, however, that dose-additive and response-additive models may yield the same answer in the very low dose region. Research in project 2 will evaluate models for assessment of mixtures of diverse chemicals, again with emphasis on the lower regions of the dose-response curves. Research in project 2 will:

- Test the null hypothesis that additivity of effects on membrane excitability will be observed when mixtures of pyrethroids and organochlorines are administered to individual cells and cells in intact brain slices;
- Test the null hypothesis that OPs and carbamates will interact in a dose-additive manner**;**
- Use genotoxicity assays to examine low dose additivity assumptions for mixtures of compounds that act via a different MOAs;
- Analyze and interpret the results of a full-factorial study (5x5x5) of the organ toxicity of three diverse chemicals, including trichloroethylene, diethylhexyl phthalate, and heptachlor, as well as all possible toxicant combinations;
- Develop and use experimental designs and statistical analysis methods for mixtures of multiple chemicals having different MOAs; and
- Test the interaction of persistent chemicals (e.g., PCBs, pesticides, polybrominated diphenyl ethers) and metals (methylmercury, organotins) that are not similar in structure and assess their effects on neuronal PKC**.**

Project 3- Examine the Usefulness of the Assumption of Additivity Under Repeated Exposure Conditions. The existing literature on the effects of chemical mixtures focuses almost exclusively on acute exposure scenarios. The results from these acute experiments (additivity, synergy, antagonism) must be used, for lack of better information, to estimate the risk of chronic exposure to these mixtures. However, it is well established in biology that receptor systems will attempt to maintain homeostasis; and, when subjected to over-activation or chronic block, receptor based systems will modulate accordingly to return the system to its previous balance. This has been observed as increases or decreases in receptor or enzyme number; up- or down-regulation of second messenger response systems coupled to receptors; and alterations in the expression of specific subunits comprising the enzyme, receptor, or ion channel. Beyond the level of the biomolecular receptor, changes in metabolism may affect the amount of active compounds in the mixture. Thus, responses which were additive following single, acute exposures, may deviate from additivity following repeated exposure to the same stimulus (mixture). Research in this project will:

- Develop models for mixtures of ChE-inhibiting pesticides in adult animals that determine the degree to which repeated exposure to ChE-inhibiting pesticides will differ from acute exposure responses and
- Determine models for mixtures of pesticides in developing animals.

Impact

The results from this research effort are expected to lead to improvements in the risk assessment of chemical mixtures by (1) testing assumptions of dose- versus response-additivity, especially in the low dose region; (2) examining changes in "additivity" from acute to chronic exposure scenarios; and (3) development of appropriate and efficient statistical models for testing the additivity

hypothesis in mixtures comprised of larger numbers of chemicals. Results from this program project will include chemical specific data, as well as new statistical methods and experimental designs for the conduct, analysis, and interpretation of mixtures experiments. In addition, the information from this research may be used to support guidance for the risk assessment based on the type of mixtures (e.g., common MOA mixtures or dissimilar MOA chemicals, simple mixtures or complex mixtures), conditions (e.g., higher dose versus low dose, acute versus chronic), and endpoints (e.g., genotoxicity, neurotoxicity, cytotoxicity) for which the default assumptions of dose-addition or response-addition have been validated.

Cross-Agency Interactions

This program project involves cross-ORD collaborations between NHEERL and NCEA explicitly in projects 1 and 2. The NCEA collaborators are Ms. L. Teuschler and Dr. R. Hertzberg. In addition to their specific work on these projects, they will participate in the program project research team meetings. The planned workshops (use of dose addition to predict interactive effects of mixtures with a common MOA; use of response addition to predict interactive effects of mixtures with diverse MOAs; effect of dose on interactive toxicity) will include experts and participants from across ORD, the Program Offices, and Regions**.**

5.5 Gap Analysis and Links to Other Multi-Year Plans

In the past several years, cumulative risk has taken on increased importance as evidenced by several recent publications such as *Pesticides in the Diets of Infants and Children* (NRC, 1993) and *Science and Judgment in Risk Assessment* (NRC, 1994). NHEERL research is consistent with research needs identified in those documents and with issues raised in the *Supplementary Guidance for Conducting Health Risk Assessment of Chemical Mixtures* (US EPA, 2001), as well as the legislative requirement of the FQPA for the Agency to focus on the cumulative effects of pesticides that have a common mechanism of toxicity. NHEERL research also focuses on more recent concerns about predicting risk from mixtures of chemicals with dissimilar mechanisms, as well as issues related to changes in mechanism as a function of repeated exposure. NHEERL research will examine mixtures from several chemical classes, including the OPs, carbamates, pyrethroids, halomethanes, persistent bioaccumulative toxicants, conazoles, and chloroatrazines. That NHEERL has little or no research planned for aggregate risk is a major gap although NHEERL will examine the validity of route-to-route extrapolation for selected air toxics within the Air Toxics MYP.

NHEERL research on cumulative risk is linked to several other MYPs. In the case of EDCs, NHEERL will examine the effects of mixtures of dioxin-like chemicals on the development in the rat and will examine the mechanism(s) by which PCB mixtures affect the development of the nervous system. In the case of Air Toxics and PM, NHEERL research on cumulative risk is linked to research to characterize effects of volatile organic compound mixtures using *in vitro* assays and studies on interaction between PM and gaseous co-pollutants in mediating COPD. NHEERL research also is linked to research to investigate the assumption of additivity in assessing pesticides with common MOAs (e.g., mixtures of OPs, pyrethroids).

5.6 References

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APPENDIX A
Resource Allocation for Program Projects
HARMONIZATION

TITLE	TEAM LEADERS	FY03 FTE (ESTIMATES)
Harmonization of Cancer and Non-cancer Risk Assessment: Disruption of Mitogen-Activated (MAPK) Signaling as a Common Mode of Action for Environmental Toxicants	**William Mundy (NTD) Barbara Abbott (RTD)**	**5.7**
Disruption of Luteinizing Hormone (LH) Secretion as a Common Mode of Action for Altered Fertility, Reproductive Disease, and Cancer of the Reproductive System	**Ralph Cooper (RTD)**	**3.1**
Modulation of Cytochrome P-450s and Other Xenobiotic Metabolizing Enzymes (XMEs) Leading to Common Mode of Action for Multiple Toxicities	**Stephen Nesnow (ECD)**	**10.0**

SUSCEPTIBLE SUBPOPULATIONS

Identifying and Validating Biologic Indicators of Susceptibility and Sensitivity among Children to Assess Potential Risk of Adverse Outcomes Associated with Environmental Exposure	**Stanley Barone (NTD) Pauline Mendola (HSD)**	**14.4**
Extrapolating Across Windows of Vulnerability to Assess Children's Health Risks Using Rodent Toxicity Data	**Hugh Barton (ETD)**	**2.7**
Long-Term Effects of the Developmental Environment	**John Rogers (RTD) Chris Lau (RTD)**	**8.5**
Susceptible Subpopulations: Susceptibility Associated with the Aged	**Andrew Geller (NTD)**	**4.2**
Environmental Risk Factors for Asthma	**Ian Gilmour (ETD)**	**4.8**
Environmental and Genetic Interactions in Hypertensive Rats: Oxidative Stress as a Common Susceptibility Attribute for Non-cancer Risks	**Urmilla Kodavanti (ETD)**	**5.9**
Genotype and Phenotype in the Metabolism, Toxicity and Carcinogenicity of Arsenic	**Kirk Kitchin (ECD) David Thomas (ETD)**	**3.3**

CUMULATIVE RISK

Exploring the Limits of Additivity	**Jane E Simmons (ETD) Stephanie Padilla (NTD)**	**7.5**

APPENDIX B

Annual Performance Goals (APGs) and Measures (APMs)
Harmonization Research

ANNUAL PERFORMANCE GOALS AND MEASURES		YEAR	Program Project
APG - Identify potential common modes of actions that underlie different toxic effects		**2003**	
APM	**Report on immunohistochemical methods for the detection of signal transduction activation *in vivo* in animals and humans exposed to pollutants**	**2003**	**PP#1 MAP-Kinase**
APM	**Report summarizing biological basis for common precursors for cancer and non-cancer effects by prototypic agents**	**2003**	**All Program Projects**
APG - Determine utility of emerging technologies in harmonizing risk assessment		**2008**	
APM	**Report on the development and application of emerging technologies to detect changes in signal transduction *in vitro* and *in vivo***	**2005**	**PP#1 MAP-Kinase**
APM	**Report on the use of toxicogenomic and related technologies to define common modes of action of P-450 modulating chemicals**	**2006**	**PP#3 P-450 and XME**
APM	**Summary report on the use of emerging technologies in risk assessment**	**2008**	**All Program Projects**
APG - Provide scientific basis for use of mechanistic data in harmonized risk assessment		**2012**	
APM	**Report on the use of mechanistic data to define common modes of action for risk assessment of P450 modulating chemicals**	**2004**	**PP#3 P-450 and XME**
APM	**Report on the range of chemicals that modify regulation of luteinizing hormone**	**2005**	**PP#2-LH**
APM	**Report on the mechanisms involved in altering luteinizing hormone**	**2006**	**PP#2-LH**
APM	**Report on determining common modes of action for developmental reproductive and neural toxicities induced by P-450 modulating chemicals**	**2006**	**PP#3 P-450 and XME**
APM	**Report summarizing use of cell signaling data as common mode of action for harmonization**	**2007**	**PP#1 MAP-Kinase**
APM	**Report on use of cell signaling data for extrapolation of mode of action information from *in vitro* to the whole animal**	**2008**	**PP#1 MAP-Kinase**
APM	**Report on the integration of toxic effects, P-450 modulation, structure-activity analysis, and gene expression profiling for application to risk assessment**	**2008**	**PP#3 P-450 and XME**
APM	**Report on characterization of cancer and non-cancer reproductive effects following modulation of luteinizing hormone secretion**	**2008**	**PP#2-LH**

ANNUAL PERFORMANCE GOALS AND MEASURES		YEAR	Program Project
APM	**Report summarizing data from prototypic compounds acting through p-450/XME modulation**	**2008**	**PP#3 P-450 and XME**
APM	**Report on evaluation of risk associated with multiple exposures to chemicals having differential effects on secretion of luteinizing hormone**	**2010**	**PP#2-LH**
APM	**Report on the use of cell signaling data for extrapolation of mode of action information for interspecies extrapolation**	**2010**	**PP#1 MAP-Kinase**
APM	**Report summarizing harmonized risk assessment for chemicals modifying luteinizing hormone secretion**	**2012**	**PP#2-LH**
APM	**Report summarizing risk assessment for chemicals acting by oxidative stress**	**2012**	**PP#9 Oxidative Stress**
APM	**Summary report on use of cell signaling data for use in harmonization of risk assessment**	**2012**	**PP#1 MAP-Kinase**
APG - Provide a framework for harmonization of extrapolation factors and mechanistic data in risk assessment		**2012**	**All Program Projects**

APPENDIX C
Annual Performance Goals (APGs) and Measures (APMs)
Susceptible Subpopulations

ANNUAL PERFORMANCE GOALS AND MEASURES		YEAR	Program Project
APG 2005	By 2005, provide risk assessors and managers with methods and tools for measuring exposure and effects in children, characterizing risk to children, and reducing risks to children in schools from harmful environmental agents to support EPA risk assessment and risk management	2005	
APM	Provide proof of concept for use of a biologically based, dose response model of a specific cellular, developmental event to predict the risk of adverse outcome (i.e., toxicant induced limb defects)	2002	PP#4 Indicators
APM	Report on the biological basis of childhood asthma	2004	PP#8 Asthma
APM	Report on the health effects associated with exposure of animals and humans with asthma to diesel exhaust particles	2004	PP#8 Asthma
APM	External review draft report on conducting risk assessments for children as a sensitive subpopulation and summary of supporting ORD research	2005	PP#4 Indicators
APM	Report on the health effects associated with exposure of exhaust particles in asthma animal model	2005	PP#8 Asthma
APM	Report on at least 1 method to test for effects in the National Children's Study, including the assessment of respiratory health outcomes such as asthma	2004	PP#4 Indicators
APM	Report on assessing health effects in children under five years of age exposed to pesticides	2004	PP#4 Indicators
APM	Report on methods to collect, store, and transport biologic specimens from surrogate tissues in humans and rodent models for protein and gene expression analysis	2004	PP#4 Indicators
APG 100	By 2009, complete two or more targeted epidemiological or exposure studies on children to test hypotheses, collect data, and validate methods and tools for measuring, characterizing, and reducing real world risks from exposures to harmful environmental agents to support EPA risk assessment and risk management	2009	
APM	Report on determinants of susceptibility to the acute respiratory health effects of combustion-related pollutants among asthmatic children in 7 US communities	2003	PP#8 Asthma
APM	Report on field ready test system based on classical conditioning for use in testing developmental neurological disorders in human infants	2004	PP#4 Indicators
APM	Peer-reviewed scientific publication on intra-urban variations in the prevalence of childhood asthma associated with intra-urban gradients of combustion-related pollutants and air toxics in El Paso, TX	2004	PP#8 Asthma

ANNUAL PERFORMANCE GOALS AND MEASURES		YEAR	Program Project
APM	**Report on the characterization of physicochemical properties of the allergenic protein from selected indoor fungi and compare to other well-characterized proteins for hazard identification**	**2006**	**PP#8 Asthma**
APM	**Report on prevalence of asthma and low pulmonary function levels among 4th and 5th grade schoolchildren in a second major urban area**	**2007**	**PP#8 Asthma**
APM	**Report on analysis of National Children's Study data on relationship between exposure to environmental agents and adverse health outcomes**	**2008**	**PP#4 Indicators**
APM	**Report on a validation of field-collected diesel exposure biomarkers**	**2008**	**PP#8 Asthma**
APG 2012	**By 2012, provide risk assessors and managers with methods and tools for measuring exposure and effects in children, including adolescents, characterizing cancer and non-cancer hazards and risk to children, and reducing risks to children in schools from harmful environmental agents to support EPA risk assessment and risk management**	**2012**	
APM	**Report on the use of genomics for monitoring expression of health status through analysis of accessible tissues and cells**	**2004**	**PP#4 Indicators**
APM	**Report on the long-term persistent effects of developmental exposure to environmental chemicals on cancer and non-cancer endpoints**	**2004**	**PP#6 Developmental Environment**
APM	**Report on proteins from several indoor fungi that pose a hazard with respect to allergenicity**	**2004**	**PP#8 Asthma**
APM	**Report on long-term effects of *in utero* insult on adult health and reproduction to assess adequacy of current testing guidelines for reproductive toxicity**	**2005**	**PP#6 Developmental Environment**
APM	**Report on the validation of a human blood ex vivo model to evaluate dose, genetic and age specific factors impacting inter- and intra-individual phenotypic response measurements**	**2006**	**PP#$ Indicators**
APM	**Report on the assessment of the relative potency of several indoor fungal allergens to obtain quantitative information for risk assessments**	**2005**	**PP#8 Asthma**
APM	**Report on age-related pharmacokinetic and pharmacodynamic changes in developing animals**	**2006**	**PP#5 Windows**
APM	**Report on the reliability of assays to measure endogenous and exogenous constituents of breast milk that are implicated in altering health status of women or their breast-fed children**	**2006**	**PP#4 Indicators**
APM	**Report on feasibility of measuring early postnatal serum measures of neurotrophic factors (NTF), neurotransmitters, and cytokines in human infants**	**2006**	**PP#4 Indicators**

ANNUAL PERFORMANCE GOALS AND MEASURES		YEAR	Program Project
APM	Report on development of principles to be used to assess cancer risks in children	2006	PP#4 Indicators
APM	Report on the application of mechanistic information in assessing cancer risk in children	2007	PP#4 Indicators
APM	Report on the mechanism of chemical-induced childhood asthma	2007	PP#8 Asthma
APM	Summary report on state of science and application to risk assessment of pharmacokinetics and pharmacodynamics as it relates to children's health	2008	PP#5 Windows
APM	Report on the predictive validity of early postnatal serum measures of neurotrophic factors (NTF), neurotransmitters, and cytokines in human infants for developmental neurological disorders	2010	PP#4 Indicators
APG 135	By 2008, provide risk assessors and managers with methods and tools for assessing differences in exposures and responses to harmful environmental agents between the elderly and younger adults.	2008	
APM	Report on modeling for age-dependent pharmacokinetics in risk assessment	2004	PP#5 Windows PP#7 Aging
APM	Report on age-dependence of protective repair and plasticity mechanisms in aged animal models	2005	PP#7 Aging
APM	Report on latent adverse health effects of developmental exposures	2005	PP#6 Developmental Environment PP#7 Aging
APM	Report on differential response of older animals and cells to environmental agents	2008	PP#7 Aging
APM	Report on gene expression changes correlated with latent adverse health effects of developmental exposures	2006	PP#6 Developmental Environment
APM	Report on mechanisms of susceptibility to environmental insult in models of aging	2010	PP#7 Aging
APG	By 2007, analyze and demonstrate the role of genetic factors in causing cancer and non-cancer endpoints	2007	
APM	Report on genetic polymorphisms that might alter human risk of arsenic carcinogenesis	2002	PP#10 Genetics
APM	Report on the role of genetic factors in cancer endpoints	2007	PP#10 Genetics
APM	Report on gene array data from animal and human polymorphisms and relation to disease susceptibility and underlying oxidative stress	2008	PP#9 Oxidative Stress
APG 126	By 2006, evaluate the variation in vulnerability to environmental agents as a result of health status as reflected by nutritional status and pre-existing disease.	2006	

ANNUAL PERFORMANCE GOALS AND MEASURES		YEAR	Program Project
APM	**Report on the effects of pre-existing respiratory disease on response to air pollutants**	**2005**	**PP#8 Asthma**
APM	**Report on comparative dose-response toxicity data for susceptible hypertensive rats and role of oxidative stress**	**2006**	**PP#9 Oxidative Stress**

APPENDIX D
Annual Performance Goals (APGs) and Measures (APMs)
Additivity

ANNUAL PERFORMANCE GOALS AND MEASURES		YEAR	Program Project
APG 134 - Develop methods and measurement data to support models of cumulative exposures, dose, and effects		**2009**	
APM	**Report on studies examining interactions of carbamate pesticides in mixtures**	**2004**	**PP#11 Additivity**
APM	**Complete analysis and report on full factorial 5x5x5 study of effects of three diverse chemicals on several non-cancer endpoints**	**2004**	**PP#11 Additivity**
APM	**Report on cumulative risk of exposure to two high use pesticides on female reproductive system**	**2004**	**PP#11 Additivity**
APM	**Report of studies on modeling of dose-response curves of prototypic chemicals having similar or different modes or mechanisms of action**	**2004**	**PP#11 Additivity**
APM	**Develop *in vitro* preparations to study interaction of pyrethrin pesticides *in vitro***	**2005**	**PP#11 Additivity**
APM	**Report on effects of disinfectant by-product mixtures *in vivo* and *in vitro***	**2005**	**PP#11 Additivity**
APM	**Develop parameters to model dose-additivity for mixtures of organophosphate and carbamate pesticides**	**2005**	**PP#11 Additivity**
APM	**Develop *in vitro* methods to predict interactions of persistent environmental toxicants**	**2005**	**PP#11 Additivity**
APM	**Demonstrate effects of pesticide mixtures after repeated dosing scenarios *in vivo* are different than those following short-term exposure**	**2006**	**PP#11 Additivity**
APM	**Report on studies to predict interactions between persistent environmental toxicants**	**2006**	**PP#11 Additivity**
APM	**Report on studies to predict interactions between environmentally relevant mixtures of pyrethrins**	**2007**	**PP#11 Additivity**
APM	**Report on prediction of interactions of chemicals *in vivo* based on principles developed *in vitro***	**2008**	**PP#11 Additivity**
APM	**Proceedings from workshop on principles of dose additivity and response-additivity to predict interactions between chemicals with similar and dissimlar modes of action, respectively**	**2008**	**PP#11 Additivity**
APM	**Summary report on methods and models to predict interactions of environmental chemicals**	**2009**	**PP#11 Additivity**
APM	**Chemical mixtures report on the use of dose-additivity to predict interactions of chemicals with a common mode of action, and use of response additivity to predict interaction of chemicals having different mechanisms of action**	**2011**	**PP#11 Additivity**

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