

PATHWAYS TO BREAST CANCER:

A CASE STUDY FOR INNOVATION IN
CHEMICAL SAFETY EVALUATION



A report of the Breast Cancer and Chemicals Policy Project, produced by the University of California, Berkeley and the Natural Resources Defense Council, with funding from the California Breast Cancer Research Program, University of California Office of the President



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Authors

Megan Schwarzman, MD, MPH
Sarah Janssen, MD, PhD, MPH

Contributors

Ruthann Rudel, MS
Lauren Zeise, PhD
Joseph Guth, PhD, JD
Gina Solomon, MD, MPH
Dale Johnson, PharmD, PhD
Shanaz Dairkee, PhD
Rachel Morello-Frosch, PhD
Suzanne Fenton, PhD
Ron Melnick, PhD
Jean Latimer, PhD
Vincent James Cogliano, PhD

Acknowledgements

Georgia Green, Suzanne Llewellyn, Dr. Ann Nardulli, Dr. Peggy Reynolds, Dr. Christina Rudén, Dr. Martha Sandy, Dr. Mike Shelby, Theresa Steuve, Catherine Thomsen, Antonio Wlasslowsky, Dr. Tracey Woodruff, Suzanne Vyborney



This project was funded by a grant from the California Breast Cancer Research Program.
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Executive Summary

Breast cancer, the most common invasive cancer in women, is hypothesized to be linked to industrial chemical exposure through the environment and the use of consumer products. A major challenge in understanding the extent to which chemicals contribute to breast cancer is a lack of toxicity information—a data gap—for tens of thousands of commonly used chemicals. Through its Green Chemistry Initiative, California is attempting to address this data gap by seeking ways to develop toxicity information for chemicals used in consumer products. A bill recently introduced in the U.S. Congress to reform the decades-old Toxic Substances Control Act (TSCA) calls for the generation and disclosure of information on the toxicity of industrial chemicals. Generating the data to inform these programs will require new, more efficient approaches to produce reliable information on the hazards posed by the tens of thousands of chemicals already in commerce.

To investigate how such efforts could help identify chemicals that may raise the risk of breast cancer, the California Breast Cancer Research Program¹ designed and funded the

Breast Cancer and Chemicals Policy (BCCP) project.² The goals of the BCCP project were three-fold:

- **Develop an approach for identifying chemicals** that may contribute to the development or progression of breast cancer,
- **Identify research needs** and recommend improvements to existing test methods, and
- **Pilot a model process** that can be applied to other disease endpoints, enabling the ultimate aim of producing a comprehensive approach for identifying hazardous chemicals.

Drawing on the fields of cancer biology, toxicology, medicine, epidemiology, public health, and public policy (*Figure 1*), a multidisciplinary expert panel (Panel) reviewed existing methods for chemical toxicity testing and developed a testing scheme, called the Hazard Identification Approach. This approach provides a methodology for the identification of substances that could elevate breast cancer risk.

The Panel's analysis followed the lead of major new initiatives in chemical hazard evaluation that seek to shift emphasis from decades-old whole animal testing protocols to more efficient *in vitro* mechanism-based chemical screening.³⁻⁷

The Panel used a four step process to achieve the stated goals. Working from current epidemiologic and laboratory evidence, the Panel first identified changes in biological processes associated with the development or progression of breast cancer. Second, they identified existing toxicity testing methods that detect these changes. Third, the Panel designed a testing scheme, calling it the Hazard Identification Approach, for identifying chemicals that may raise the risk of breast cancer. The panel also recommended ways of prioritizing the types of chemicals that would undergo testing. The fourth step was to conduct a virtual pilot test of the recommended Hazard Identification Approach. Following is a description of each step.

1. Identification of biological processes associated with breast cancer

The Panel determined toxicity endpoints: alterations to biological processes associated with the development, progression, or susceptibility to breast cancer. These toxicity endpoints were divided into three categories (Figure 6):

- Cellular and molecular mechanisms, (e.g., activity at hormone receptors),
- Tissue changes (e.g., altered mammary gland development), and
- Susceptibility factors (e.g., early puberty).

Within each category, the Panel identified distinct biological endpoints that could be evaluated in a toxicity test.

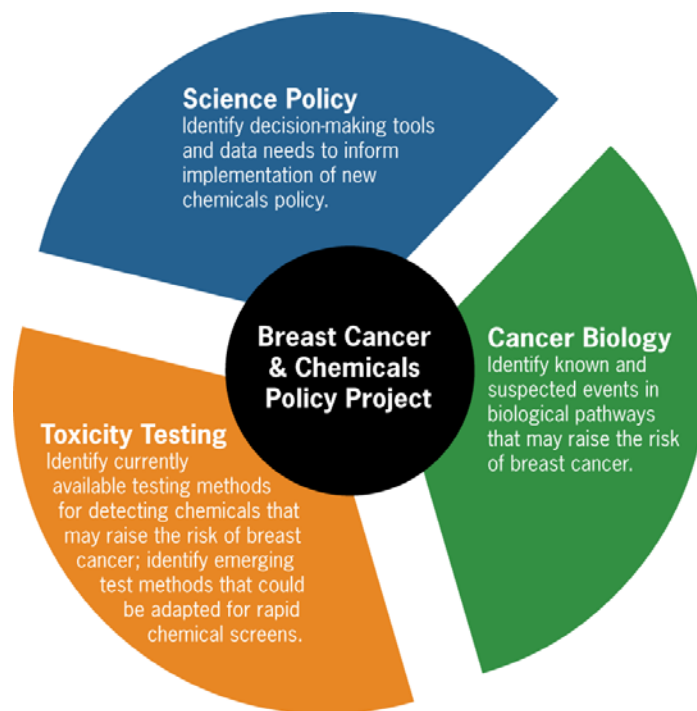


Figure 1. Framework of the Breast Cancer and Chemicals Policy Project. The Breast Cancer and Chemicals Policy Project was conducted by a multidisciplinary panel consisting of experts in toxicology, cell and mammalian biology, medicine, epidemiology, endocrine disruption, environmental justice, science policy and breast cancer advocacy. The Panel developed a method for identifying, prioritizing, and testing chemicals that may raise the risk of breast cancer.

2. Identification of toxicity testing assays for evaluating chemicals

The Panel identified examples of computational (*in silico*), *in vitro*, *in vivo*, and epidemiological methods for evaluating a chemical's ability to alter biological processes relevant to breast cancer. Validated assays were catalogued, as were those that could be validated in the near future (based on their current use in laboratory research), and those that are emerging from high throughput toxicity testing methods.[†]

* Computational toxicology, as defined by U.S. EPA (U.S. EPA, 2003) "the application of mathematical and computer models to predict the effect of an environmental agent and elucidate the cascade of events that result in an adverse response."

† A National Academy of Sciences committee (NAS/NRC 2007) defined high-throughput testing as "efficiently designed experiments that can be automated and rapidly performed to measure the effect of substances on a biologic process of interest. These assays can evaluate hundreds to many thousands of chemicals over a wide concentration range to identify chemical actions on gene, pathway, and cell function."

3a. Propose methods for setting priorities

As tens of thousands of largely untested chemicals are considered for toxicity testing, substances should be prioritized based on preliminary indicators of hazard (e.g., potential estrogenic activity). Additionally, substances to which people are likely to be exposed, which have physical or chemical properties of concern (such as persistence or bioaccumulative potential), or which have been flagged by computational methods—should be prioritized for evaluation of any potential harmful human health effects, not only breast cancer.

3b. Designing an overall Hazard Identification Approach

The Panel designed a testing scheme for identifying chemicals that may raise the risk of breast cancer. This testing scheme is the Panels' Hazard Identification Approach (Figure 7), which recommends testing chemicals for their potential to increase breast cancer risk through any of the following mechanisms:

- Mechanisms associated with carcinogenesis in general, including cell cycle changes and genotoxicity,
- Mechanisms associated with endocrine disruption, and
- Altered development and maturation of the mammary gland.

4. Pilot testing the Hazard Identification Approach

The panel conducted a virtual validation of the proposed Hazard Identification Approach—a pilot test of 20 substances for which sufficient animal or human data exist to characterize their links to breast cancer. This pilot test consisted of a literature review, searching for results of toxicity tests from the Panel's Hazard Identification Approach. The findings of this pilot test will be published separately in a peer-reviewed publication.

Chemical toxicity testing—and the public policies that require it—can be critical tools in breast cancer prevention, providing a practical basis for reducing potentially harmful exposures.

Recommendations of the Breast Cancer and Chemicals Policy Project

Based upon their expert consensus and preliminary assessment, the Panel recommends the following approach to toxicity testing to increase its relevance to breast cancer:

1. Chemicals used in industrial processes or found in the environment, consumer products, or workplaces must be tested for their possible impact on breast cancer risk. Testing should identify alterations in biological processes relevant to breast cancer, including:

- Cell cycle changes,
- Genotoxicity,
- Endocrine disruption (estrogenicity and other hormonal effects), and
- Mechanisms associated with altered mammary gland development or maturation.

2. To accurately evaluate the potential of a chemical to raise the risk of breast cancer, toxicity tests must be designed and conducted with the understanding that effects vary depending on *timing of exposure* and *underlying susceptibility factors*. To account for this, toxicity tests need to:

- Assess the impact of chemical exposure during a variety of life stages, including gestation, puberty, pregnancy, and post-menopause; and
- Account for increased susceptibility due to genetic variation, underlying disease, or exposure to other chemicals and environmental stressors.

3. New research is needed to improve the scientific tools available to identify chemicals that contribute to breast cancer risk. This includes:

- Further investigation of the biological processes that, when altered, increase the risk of breast cancer;
- Development and validation of new toxicity testing methods, including high-throughput screening, to detect chemicals that alter relevant biological processes;
- Adaptation of current toxicity testing methods to more specifically address mechanisms relevant to breast cancer; and
- Interdisciplinary efforts to link current knowledge of breast cancer etiology with the design and implementation of chemical toxicity tests.

4. A similar process as that used by the Panel should be used to develop testing methods specific to other diseases. In practice, a comprehensive approach to identifying chemicals that may pose a human health hazard is necessary to generate information for regulatory agencies as well as chemical producers and end users.

Conclusions

Chemical toxicity testing—and the public policies that require it—can be critical tools in breast cancer prevention, providing a practical basis for reducing potentially harmful exposures.^{8,9} The Hazard Identification Approach developed by the Panel can guide the development of toxicity testing specific to breast cancer. Information generated by implementing the Hazard Identification Approach could a) increase the relevance of chemical assessments for public health; b) provide a scientific basis for identifying and

prioritizing chemicals that may increase breast cancer risk; and c) generate data to support use of less toxic alternatives.

More comprehensive and efficient detection of chemicals linked to breast cancer will require both ongoing research into the biological basis of breast cancer and development of new toxicity testing methods, particularly the development of *in vitro* chemical screening techniques and high-throughput methods.

Meanwhile, it is essential that practical approaches to identifying potential breast carcinogens are implemented now, to begin addressing the backlog of untested chemicals and inform the development of new chemicals policies. These approaches should include use of currently available methods (e.g. tests for estrogen-like effects or genotoxicity), as well as the adaptation of existing tests to include endpoints relevant to breast cancer. For example, the OECD extended-one generation assay currently used in international toxicity testing guidelines could easily be modified to include an evaluation of changes to mammary gland development after chemical exposure.¹⁰

When fully developed, the Hazard Identification Approach recommended by the Panel has the potential to generate toxicity information useful to consumers, workers, product manufacturers, chemical producers, and policy makers. Applied to large numbers of chemicals, this could greatly improve our ability to focus the lengthiest and most expensive tests on chemicals with the highest potential for increasing the risk of breast cancer or other diseases. Ultimately, this should lead to the ability to identify and use the least toxic chemical alternatives.

I. Introduction

Over the last century, chemicals have become the material basis of industrialized societies. In 2006, more than 34 million metric tons of chemicals were produced in, or imported into, the United States every day.¹¹ Over the next quarter-century, global chemical production is projected to double, rapidly outpacing the rate of population growth.¹² Hundreds of chemicals are routinely detected in people and in ecosystems worldwide, yet the health and environmental effects of the vast majority of these substances are poorly understood.

New chemicals laws recently implemented in Europe and Canada, and under development in California and the U.S., aim to increase the generation and public disclosure of information on the adverse effects to human health and the environment of chemicals used in products, workplaces and manufacturing processes.¹³ As new chemical information requirements are codified into law, several urgent questions have emerged:

- Are test methods available that can be efficiently applied to the thousands of untested chemicals already in commerce?
- How can existing toxicity tests be assembled to identify chemicals of concern?
- Does newly emerging science offer test methods that are cost-effective and yet provide useful hazard information?
- What is the highest priority research needed to improve chemical screening?¹⁴

To investigate these questions through the lens of a prevalent disease, the California Breast Cancer Research Program¹⁵ designed and funded the Breast Cancer and Chemicals Policy (BCCP) project.¹⁶ The project was conducted by a multidisciplinary expert panel (Panel).

Hundreds of chemicals are routinely detected in people and in ecosystems worldwide, yet the health and environmental effects of the vast majority of these substances are poorly understood.

The goals of the BCCP project were three-fold:

1. **Develop an approach for identifying chemicals** that may contribute to the development or progression of breast cancer,
2. **Identify research needs** and recommend improvements to existing test methods, and
3. **Pilot a model process** that can be applied to other disease endpoints, enabling the ultimate aim of producing a comprehensive approach for identifying hazardous chemicals.

The primary outcome of the BCCP project was the design of a chemical testing scheme, called the Hazard Identification Approach. The Hazard Identification Approach is a recommended method for testing a chemical's effect on a variety of endpoints in biological processes that, if altered, could affect breast cancer risk. The approach is designed to utilize existing test methods that are widely available and, where possible, validated. Ultimately, new toxicity testing paradigms and data requirements must be able to detect many potential effects of chemicals on human health and the environment, making this single disease-based approach a starting point for development of comprehensive chemical testing. Ideally, a unified approach will be implemented to screen many chemicals, identify those that merit immediate action, those that require further testing, and those that may serve as safer alternatives. The toxicity information generated by such testing should be relevant to consumers, product manufacturers, workers, chemical producers, and regulators.

The Panel developed this approach within the context of major new initiatives in chemical hazard evaluation that seek to shift emphasis from expensive, decades-old, whole animal test protocols to more efficient *in vivo* and *in vitro* mechanism-based chemical screening. A recent study by the National Academy of Sciences (NAS) recommended screening chemicals based on toxicity pathways, rather than relying on traditional toxicology or epidemiologic studies that focus exclusively on observations of apical, or overt, disease endpoints, such as the development of a tumor, birth defect, or infertility.¹⁷

Examining the biological processes that lie along the pathway between exposure and disease

facilitates identification of early indicators of harm, such as interference with cellular signaling, hormone disruption, or alterations in gene expression. These indicators occur “upstream” of apical endpoints and can potentially be evaluated using cell-based tests in place of laboratory animals. Many of the methods needed to implement the shift toward efficient chemical screening are still under development. By necessity, therefore, the Panel’s recommendations draw from existing toxicity testing assays—some of which are based on traditional whole animal models—while pointing toward the future use of cell-based testing and high-throughput methods that can screen hundreds of chemicals for upstream indicators of disease.

II. Background

A. Why breast cancer?

Cancer is the second leading cause of death in the United States,¹⁸ and breast cancer is the most common invasive cancer and the leading cause of death in American women in their late 30s to early 50s.¹⁹ Breast cancer rates are highest in developed countries. In 2006, an estimated one in eight U.S. women would develop breast cancer during her lifetime.²⁰ Significant racial disparities exist in breast cancer incidence: in 2007, the breast cancer death rate for women aged 45--64 years was 60% higher for black women than white women (56.8 and 35.6 deaths per 100,000, respectively).²¹

Although there has been a recent decline in U.S. breast cancer incidence rates, presumably due to a decrease in the use of hormone replacement therapy,^{22 23} premenopausal breast cancer incidence is reported to be on the rise.²⁴

Many factors influence trends in breast cancer incidence, such as increased screening rates, obesity, and delayed childbearing. It is impossible to attribute geographic and temporal trends to any particular known risk factor. Although clinical have improved breast cancer survival rates, the ideal would be to prevent the disease from ever occurring.

“[Breast cancer] incidence has stabilized in the U.S., but it has stabilized at one of the highest rates in the world, and as women move from lower risk regions of the world to the U.S., their incidence goes up and continues to rise over a couple of generations. So we know that that’s not genes and there’s something about industrial society that’s playing an important role.”

-- Julia Brody, President’s Cancer Panel report.

Among the suspected and preventable risk factors for breast cancer is exposure to chemicals in the environment. While it is difficult to determine the proportion of breast cancer attributable to environmental pollutants, inherited risk factors only explain an estimated 5-10%, or at most 27%, of breast cancer cases.²⁵ Evidence for the role of some common chemicals and environmental contaminants in breast cancer includes observations of chemically-induced mammary tumors in laboratory animals.²⁷ In addition, many chemicals alter hormone signaling systems (e.g. estrogen) that play a role in governing susceptibility to breast cancer or in the development and progression of the disease. Because breast cancer is a significant source of morbidity and mortality in the U.S., and chemical exposures are so common, the public health impact of reducing exposures could be profound, even if the true relative risk posed by chemicals is modest.²⁸

Established environmental causes of breast cancer include estrogenic compounds (e.g., hormone replacement therapy²⁹ and diethylstilbestrol (DES)³⁰), other substances with hormonal effects (e.g., alcohol), and some agents that cause direct genetic damage (e.g., ionizing radiation).^{31 32} Experimental models, however, raise concern for a much broader list of chemicals. More than 200 compounds have been found to induce mammary tumors in animals, including vinyl chloride, 1,3-butadiene, and acrylamide, but there is little data on their effects in women. While hormonal factors are among the most apparent causes of breast cancer and should be a major focus of toxicity testing, strategies for identifying potential breast carcinogens must also consider mutagenicity (e.g., ionizing radiation) and other mechanisms of carcinogenesis.³³

B. Policy Context

To date, flawed environmental laws have played a significant role in permitting hazardous chemicals to persist in commerce.³⁴⁻³⁸

Several key deficiencies afflict the primary U.S. law governing industrial chemicals, the Toxics Substances Control Act (TSCA). First, TSCA imposes on the government the burden of proving a chemical presents an unreasonable health or environmental risk before it can regulate that chemical. Equally important is a second deficiency: TSCA does not require manufacturers to provide sufficient toxicity information about their chemical products for government or the public to determine whether or not the chemicals are hazardous. Of the rudimentary toxicity information that must be submitted to the government for new chemicals, no data regarding hormone disruption or breast cancer potential is required, and only a fraction of the information must be publicly available.³⁹

The resulting data gap has made it impossible for consumers to determine whether most chemicals or products containing them are safe and has often left government with insufficient information to protect public health or the environment. Without this information, neither commercial nor individual consumers can identify safer substances. The absence of data requirements discourages industry from testing their products, and undermines any market incentive to develop safer products. Taken together, these factors constitute significant barriers to the identification of hazardous substances and development and marketing of safer alternatives. In the context of pesticide regulation, U.S. EPA has recently acknowledged the critical role of information disclosure in enabling consumers to choose less hazardous products.⁴⁰

Accordingly, one of the central goals of new chemicals policies is the requirement that

manufacturers provide information on the hazardous properties of their chemicals.^{41 42}

This requirement is at the core of the European Union's landmark 2006 chemicals regulation on the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH). The law specifies information that manufacturers must provide as a condition of selling their products in Europe.⁴³ Similar requirements are proposed in the U.S. under new federal TSCA reform legislation introduced in 2010.⁴⁴

In 2007, California, ahead of the federal government, passed two laws (AB 1879 and SB 509) as cornerstones of the state's ongoing Green Chemistry Initiative. One of the key elements of this Initiative and the related laws is an effort to close the data gap by requiring chemical producers to provide more information to regulators, commercial users, and the public.⁴⁵

The BCCP project seeks to inform implementation of these laws by offering an approach to identifying chemicals that contribute to cancer, specifically breast cancer.

C. Current State of Regulatory Toxicity Testing

Carcinogens are typically identified through data from some combination of animal bioassays, epidemiologic research, and studies of specific mechanisms of action. Several U.S. governmental and international agencies compile formal lists of carcinogens.⁴⁶⁻⁴⁸ These lists are generally tiered according to the strength of scientific evidence using both animal and human data. Each agency has slightly different criteria and classification nomenclature for their categories, such as a "known human carcinogen" or "possible human carcinogen" (*Figure 2*). Some substances are deemed unlikely to be carcinogenic, and many are unclassifiable due to insufficient evidence.

Transforming Toxicity Testing

The current approach to chemical toxicity testing in the U.S. is too limited for the vast majority of chemicals in commerce, including those commonly used in consumer products. Although some chemicals are more rigorously tested, such as food-use pesticides, the available methods are too time-consuming and resource-intensive for practical application to the tens of thousands of previously untested industrial chemicals. A standard toxicity testing battery for a pesticide costs \$5-10 million.

It is evident there is a need for a new generation of toxicity testing methods to enable more efficient chemical screening.⁴⁹ A committee of the National Academy of Sciences (NAS) addressed this question in 2007, reviewing both the established and newly emerging toxicity testing methods and strategies.⁵⁰ Noting the large number of chemicals to be tested, the numerous health outcomes of concern, and the need to assess impacts on multiple life stages, the NAS committee concluded that “a transformative paradigm shift is needed” in toxicity testing. The NAS envisioned a new system to detect “upstream events”—early changes in biological processes linked to development of disease.

The committee’s report underscored the need to develop methods for detecting these upstream events (e.g., disruption of estrogen signaling), which can lead to abnormal cell proliferation and tissue growth, with the potential for progression to cancer. Consistent with the NAS recommendations, several federal research initiatives, including EPA’s ToxCast⁵¹ the NTP’s High Throughput Screening (HTS) Initiative⁵² and the interagency Tox 21 initiative⁵³, are investigating the use of HTS methods for hazard identification.

The long-term strategy for realizing the NAS vision involves a substantial multidisciplinary research program. Further strategies could build on pharmaceutical testing protocols that use HTS assays to identify potentially toxic compounds during initial drug development. Until new methods are available, it is critical to employ the current methods to begin addressing the backlog of untested industrial chemicals and the data needs of new chemicals policies. This has been the focus of the BCCP project.

While existing federal laws require little toxicity information for the majority of chemicals in commerce, some groups of chemical compounds are more closely regulated. Pharmaceuticals and pesticides, for example, must be tested prior to approval or use. The toxicity test methods and endpoints assessed can inform the development of a toxicity testing approach for untested chemicals, with a focus on a disease endpoint such as breast cancer. It is worth noting, however, that there is no requirement that toxicity test results for pharmaceuticals or pesticides be published or made publicly available. This limits the ability of consumers and the scientific community to understand the possible harmful effects of these chemicals and make informed choices.

For pesticides used on food crops, acute, sub-chronic and chronic toxicity tests are performed in rodents, to examine general toxicity, carcinogenicity, neurotoxicity, immunotoxicity, developmental, and reproductive outcomes.⁵⁴ *In vitro* tests for mutations in mammalian cells and bacteria are also required. Further tests can be performed to understand a chemical's potency, to investigate the accuracy of extrapolating animal test results to humans, or to explore potential toxicities and exposure pathways not sufficiently addressed in standard toxicity testing. Several validated *in vitro* and *in vivo* tests of a chemical's endocrine disrupting potential (e.g. effects on estrogen, androgen or thyroid hormonal systems) are now being used to screen pesticides and pesticide "inert" ingredients for these effects.⁵⁵

Carcinogen Classification by International and Federal Agencies

Direct Human	Direct Animal	Mechanistic / Indirect	IARC	U.S. EPA	U.S. NTP
Sufficient	--	--	Carcinogenic to humans (Group 1)	Carcinogenic to humans	Known to be human carcinogen
Limited	Sufficient	Strong human mechanistic			
Limited	Sufficient	--	Probably carcinogenic to humans (Group 2A)	Likely to be carcinogenic to humans	Reasonably anticipated to be a human carcinogen
Inadequate	Sufficient	Strong			
Inadequate	Sufficient	--	Possibly carcinogenic to humans (Group 2B)		
Inadequate	Limited	Strong			
Limited	Limited	--			
Inadequate	Inadequate	Strong & same class as other carcinogens			
Inadequate	Inadequate	Strong			
Inadequate	Limited	--	Not classifiable	Inadequate Information to Assess	
Inadequate	Limited	--	Not classifiable	Suggestive	(No statement)

Figure 2. Levels of Evidence for Classifying Carcinogens. A comparison of the levels of evidence used by three different governmental bodies in classifying chemicals for carcinogenic potential. Each agency publishes detailed criteria for these categories which can be found on their respective websites. IARC = International Agency for Research on Cancer. NTP = U.S. Department of Health and Human Services, National Toxicology Program. EPA = U.S. Environmental Protection Agency (Source: Lauren Zeise, presentation to Breast Cancer and Chemicals Policy Project Expert Panel, September, 2009)

Critical periods of mammary gland development

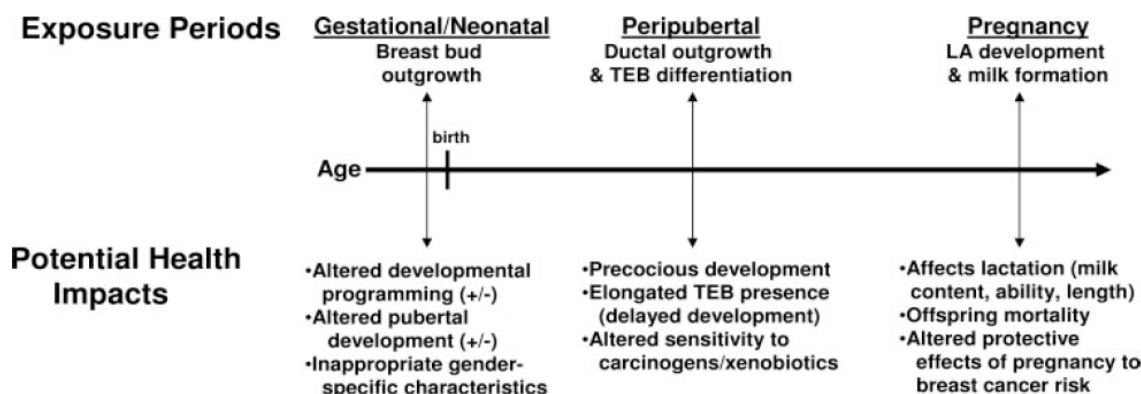


Figure 3. Timeline highlighting the critical periods of mammary gland development in rodents and the potential health impacts of chemical exposure during these developmental periods. “TEB” = terminal end bud. “LA” = Lobuloalveolar. “ +/- ” = precocious or delayed (Taken from S.E. Fenton, 2006 *Endocrinology*. 147 (Supplement):S18-34.)

Animal testing is generally used for predicting human toxicity because it is neither ethical nor practical to expose humans to chemicals for experimental purposes. Currently, regulation of chemicals shown to cause cancer does not assume concordance of tumor sites and types across species. In animal studies, the finding of a tumor in one organ system is assumed to indicate overall potential for human carcinogenicity. Compounds with potential pharmaceutical applications are often abandoned based on findings of harm (including cancer) in animal studies, and numerous pesticides are regulated on the basis of similar findings.

D. Defining and Identifying Carcinogens

A carcinogen is generally defined as a substance or agent that causes cancer, but this standard definition does not further define what it means to “cause cancer.” The International Agency for Research on Cancer (IARC) deems an agent carcinogenic if it is “capable of increasing the incidence of malignant neoplasms, reducing their latency, or increasing their severity or multiplicity.”⁵⁶

Current theories of carcinogenesis describe a multi-step process involving gene mutations or non-mutational changes to gene expression, and the resulting alterations in cellular function, tissue structure, and immune response.⁵⁷ A compound that causes cancer can act alone or

in combination with other factors at a variety of points in a biological chain of events leading to tumor formation. Substances should therefore be considered carcinogenic based on an ability to contribute to cancer risk either by directly triggering an event that leads to tumor formation (e.g. a gene mutation), or by increasing susceptibility to cancer, even if the substance does not directly induce tumors in animals or humans.

Current assessments of potential chemical carcinogens typically rely on human epidemiologic and/or laboratory animal studies for evidence of tumor formation in response to exposure. These types of studies, however, would not detect chemicals that indirectly contribute to human cancer risk by increasing disease susceptibility. The Panel therefore used the IARC definition of carcinogen broadly, acknowledging that chemicals may act as carcinogens both directly and indirectly. This definition formed the basis for identification of biological processes relevant to breast cancer.

E. Factors Affecting Breast Cancer Risk

i. Addressing Developmental Stage

Because the mammary gland undergoes significant developmental changes both before and after birth, the tissue is highly susceptible to chemical exposures during multiple critical developmental stages, including gestation,

puberty, and pregnancy (*Figure 3*). In considering experimental models for breast cancer, it is critical to address the influence of ovarian, pituitary, and placental hormones, among other factors, as well as life-stage and reproductive events, given their role modifying the susceptibility of the mammary gland to cancer.⁵⁸ Both animal and human evidence suggests that chemical exposure during critical windows of development can induce abnormal mammary development and increase breast cancer risk.⁵⁹⁻⁶³

In laboratory studies, rodent mammary gland development has been well-characterized and shares features with human mammary gland development (*Figure 4*).⁶⁴ Many research scientists rely on rodent models when investigating a chemical's toxicity and assume this has relevance for human health. A recent multi-disciplinary workshop of over 70 experts established a majority opinion that: a) the rodent model is valid for studying development

of the human mammary gland and b) mammary gland developmental endpoints are sensitive endpoints for endocrine disruption.⁶⁵

ii. Disparities in Exposure and Disease

Breast cancer incidence varies markedly in the U.S., with up to a three-fold variation solely on the basis of racial or ethnic classification.⁶⁶ Breast cancer is the number one cause of cancer death in Hispanic women and is second only to lung cancer as a cause of death from cancer in Caucasian, African American, and Indian/Alaska Native women.⁶⁷ African-American women under the age of 40 have a higher risk of breast cancer than Caucasian women of a similar age, although this trend reverses after age 40.^{68 69} Clinical outcomes also vary with race. Despite the lower breast cancer incidence in African American women over 40, they are nevertheless more likely to be diagnosed with larger tumors and to die from the disease, compared to Caucasian women.⁷⁰⁻⁷²

Developmental Events in Human and Rodent Mammary Tissue

Developmental Event	Human	Rodent
milk streak evident	EW4-6	GD10-11 (mice)
mammary epithelial bud forms	EW10-13	GD12-14 (mice), GD 14-16 (rat)
female nipple and areola form	EW12-16	GD18 (mice)/GD20 (rat)
branching and canalization of epithelium	EW20-32	GD16 to birth (mice), GD 18 to birth (rat)
secretion is possible	EW32-40 (ability lost postnatally)	at birth, with hormonal stimuli
isometric development of ducts	birth to puberty	birth to puberty
TEBs present (peri-pubertal)	8-13 year old girls	23 to 60 days old (rodents)
formation of lobular units	EW32-40, or within 1-2 yr. of first menstrual cycle	puberty and into adulthood

TEB=terminal end bud, EW=embryonic week, GD=gestational day
taken from S.E. Fenton, 2006 *Endocrinology* 147(Supplement):S18-S24.

Figure 4. Timing of mammary tissue development in humans compared to rodent species.

This variation in tumor biology and disease outcome may reflect different genetic susceptibilities within and among populations, or different exposures to environmental contaminants and other substances (e.g., DES or hormone replacement therapy). Different classes of chemical carcinogens may produce different molecular subtypes of breast cancer. Alternately, dramatic variations observed in phenotype and behavior between low risk, indolent tumors and highly aggressive lesions might reflect low vs. high dose, or sporadic vs. sustained exposure to the same carcinogens.⁷³

While there are clear racial and socioeconomic disparities in exposure to chemicals in the workplace or living environment,⁷⁴⁻⁷⁶ these have not been extensively examined in relation to racial/ethnic differences in breast cancer. The regional variations observed in breast cancer incidence, with rates highest in urban and industrialized areas, suggest a potential role for chemical exposures associated with those environments.

Early onset of puberty—especially early menarche—is a well-established risk factor for breast cancer.⁷⁷⁻⁷⁸ Thus, environmental factors that hasten the onset of sexual maturation may contribute to breast cancer risk. For example, some researchers have posited that greater use of estrogen- or placenta-containing hair care products may be contributing to the decreasing average age of puberty among African-American girls.⁷⁹ If so, these products may also contribute to racial disparities in breast cancer. Chemicals in the physical environment may contribute to breast cancer risk if they shorten human gestation, lower birth weight, or increase the risk for obesity and insulin dysregulation. All of these conditions are associated with earlier sexual maturation in girls and disproportionately affect African American women.⁸⁰⁻⁸²

Examining potential links between disparities in chemical exposures in diverse environments with racial differences in the burden of breast cancer may be relevant to understanding the higher disease incidence among young African American women and worse survival rates among some minority and low income women.

iii. Breast Cancer as a Heterogeneous Disease

Breast cancer is not a single disease. As noted above, variations in both biological and clinical presentations are associated with differences such as stage at the time of diagnosis and survival rates.

While the prevalence of some tumor types differ by race (e.g., estrogen receptor status⁸³), studies that examine the combined effect of race and socioeconomic status have been mixed in determining whether race is a significant predictor of breast cancer prognosis independent of socioeconomic status.⁸⁴⁻⁸⁶

In the past, these racial/ethnic differences in breast cancer have been largely attributed to lower income or inadequate access to medical care, including breast cancer screening and treatment. Yet African American women have significantly lower survival rates than Caucasian, Hispanic, and Asian women of similar socioeconomic status, despite receiving equivalent medical treatment.⁸⁷

In addition to observed racial differences in the incidence and severity of breast cancer, there is genetic and epigenetic heterogeneity in tumors at the tissue level, which corresponds to different clinical outcomes even within relatively homogeneous racial groups. Underlying such marked differences in tumor type and patient prognosis are consistent patterns of global gene expression.⁸⁸ These suggest that breast tumors can be categorized into a variety of molecular subtypes, accounting for heterogeneity in the disease.

III. Methods

The BCCP project drew on knowledge of cancer biology, toxicity testing and science policy (Figure 1) with an 18 member expert panel representing the fields of toxicology, cell and mammalian biology, medicine, epidemiology, endocrine disruption, environmental justice, risk assessment, science policy and breast cancer advocacy. (Appendix 1)

A Core Panel, consisting of four of the panel experts from the San Francisco Bay Area, advised the project co-directors in monthly meetings and was integral to the development and completion of the BCCP project. In addition, several graduate and undergraduate students assisted with focused research topics. Over the course of the year-long project, the entire expert Panel met twice in San Francisco for day-and-a-half long meetings. The Panel developed and implemented a four step approach to the project depicted in Figure 5.

1. Identification of biological processes associated with breast cancer

Based on current scientific knowledge, the Panel first identified “toxicity endpoints”: alterations to biological processes associated with the development, progression, or susceptibility to breast cancer. These endpoints were divided into three categories: cellular and molecular events, tissue changes, and susceptibility factors.

2. Identification of toxicity testing assays

Next, the Panel catalogued existing test methods or assays capable of screening chemicals for their ability to alter or perturb the biological processes identified in step one.

These include tests performed *in silico*, for example by computational Quantitative Structure Activity Relationship (QSAR), assays conducted *in vitro* or *in vivo*, and epidemiologic studies. Currently available, validated assays were listed, as well as those that could be easily validated (based on their current use in laboratory research). Assays used by individual labs and emerging high-throughput toxicity tests were also identified. The assays were organized into a matrix—a table that organized toxicity testing assays by the endpoints they evaluate. This matrix served as a working document used by the Panel for subsequent steps in the project (view the matrix at <http://coeh.berkeley.edu/greenchemistry/cbcrpdocs/matrix.pdf>).

3a. Proposing methods for setting priorities

For the potential tens of thousands of chemicals that have largely been untested, there needs to be a method for choosing where to start. The Panel created a set of criteria for prioritizing chemicals to undergo toxicity testing using the recommended approach.

3b. Designing an overall Hazard Identification Approach

A testing scheme, called the “Hazard Identification Approach,” was developed using existing toxicological assays, identified in Step 2 (above). The approach is designed to detect a chemical’s effect on key events within biological processes known or suspected to be linked to breast cancer. Because toxicity testing methods are rapidly evolving, the Panel chose toxicity endpoints for which chemicals should be tested, rather than specifying particular assays to use.

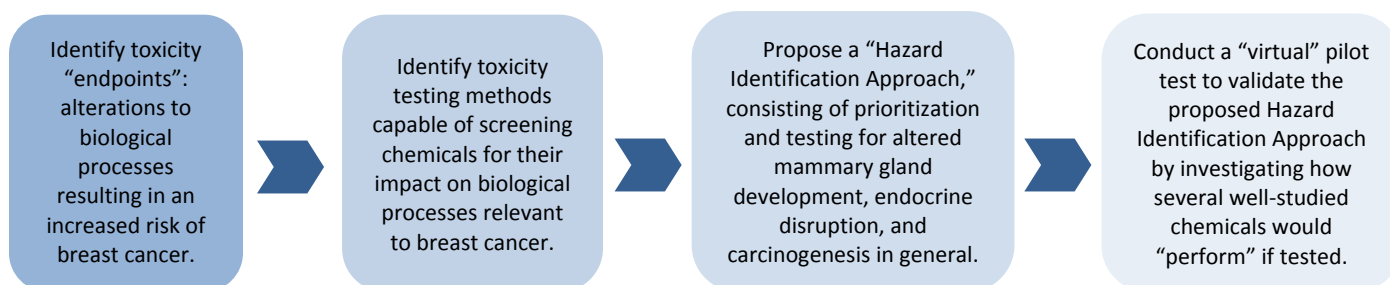


Figure 5. Four steps of the Breast Cancer and Chemicals Policy Project

The Hazard Identification Approach was designed to accommodate improved or new test methods that will undoubtedly be developed both in the near and far term.

4. Pilot testing the Hazard Identification Approach

The Panel identified 20 substances (e.g., alcohol, DES, epoxides) for which sufficient animal or human data exist to characterize their links to breast cancer. A literature review was conducted as a “virtual” pilot test of the Hazard Identification Approach by searching for the results of assays corresponding to each of the endpoints recommended by the Panel. The findings of this pilot test will be published separately, including a discussion of the potential for such an approach to produce false negative or false positive results. In general, the findings support the validity of the Hazard Identification Approach developed by the panel. Significantly more evidence was available, however, for validation of the genotoxicity outcomes than for endocrine disruption.⁸⁹

IV. Results

The results of the first three steps of the BCCP project are described below; results of the fourth step, a virtual pilot test of the Hazard Identification Approach, will be published separately. The Panel’s analysis of critical gaps in available methods is presented in the discussion section with recommendations for improved methods to detect chemicals associated with breast cancer.

Step 1. Identification of Biological Processes Associated with Breast Cancer

In keeping with the NAS’ recommendations to identify toxicity pathways rather than “apical” disease endpoints, the Panel identified biological processes associated with the development, progression, or susceptibility to breast cancer. These processes were grouped into three categories: *cellular and molecular events*, *tissue changes*, and *susceptibility factors*. Within each category, the Panel identified biological endpoints that could be

evaluated in a toxicity test. *Figure 6* is a representation of some of the biological processes identified by the Panel.

Cellular and molecular events: These effects at the cellular level include alterations in hormone levels, metabolism or receptors; changes in transcription and translation of genes associated with breast cancer; cell cycle changes; and genotoxicity. Immune modulation and oxidative stress were also identified as cellular events that may be associated with breast cancer risk. Hallmarks of cancer progression that occur at the cellular level include limitless replication potential, evasion of apoptosis, and self-sufficiency in growth.^{90 91} These changes could serve as “upstream” events or early indicators of an increased risk of breast cancer.

Tissue changes: Tissue-level changes that are phenotypic indicators of an increased risk of breast cancer include altered development of the mammary tissue, such as terminal end bud proliferation. Other tissue-level changes include increased breast density, adenomas, ductal hyperplasia, atypical hyperplasia, and carcinoma *in situ*. Other pathological findings serve as hallmarks of cancer, such as tissue invasion and sustained angiogenesis.

Susceptibility factors: An increased susceptibility to breast cancer may occur due to a number of physiological factors. These include an early onset of puberty, increased lifetime duration of estrogen exposure (early menarche or late menopause), alterations in cyclicity, changes in enzyme metabolism (genetic polymorphisms), and obesity. Taken together, these factors shed light on mechanisms of action and accompanying toxicity endpoints useful for identifying chemicals that may increase breast cancer risk.

Step 2. Identification of Toxicity Testing Assays

For each of the biological changes relevant to breast cancer listed above, the Panel catalogued examples of currently available assays capable of detecting chemicals that can induce those changes. These test methods

include computational, *in vitro*, *in vivo*, and human epidemiologic methods.

For example, cell-based (*in vitro*) assays can assess changes in cell cycle using currently available laboratory assays for apoptosis or cell proliferation; hormonal interference causing alterations in female cyclicity can be assessed by doing vaginal smears in laboratory animals to determine estrous status; and pathological changes in cells can be assessed by looking at immuno-histochemical markers of cell adhesion

or differentiation, (e.g. E-cadherin, cytokeratins) or by assessing nucleus-to-cytoplasm ratio. Additionally, whole animal (*in vivo*) assays can evaluate altered mammary gland development using whole mounts, or by measuring hormone receptor levels in animals exposed to chemicals early in life.^{92 93}

The catalogued test methods were organized into a matrix-- a table that identified assays associated with the biological endpoints identified in Step 1. This matrix served as a

Events in biological processes potentially associated with breast cancer

Cellular & Molecular Events	
Alterations in hormone levels, metabolism or receptors	Genotoxicity
Changes in gene transcription & translation	Oxidative stress
Cell cycle changes	Immune modulation
Peptide hormones (growth hormones)	Limitless replication potential
	Evasion of apoptosis
	Self-sufficiency in growth

Tissue Changes	
Breast density	TEB proliferation
Tissue invasion	Altered mammary gland development
Sustained angiogenesis	Ductal hyperplasia
	Atypical hyperplasia

Susceptibility Factors	
Obesity	Genetic polymorphisms in metabolizing enzymes
Early onset of breast development	Duration of lifetime estrogen exposure
Alterations in cyclicity	

Figure 6. Events within biological processes potentially associated with breast cancer. TEB = terminal end bud

working document that the Panel used as a reference for developing subsequent steps of the project. (View at <http://coeh.berkeley.edu/greenchemistry/cbcrpdocs/matrix.pdf>).

Step 3. Designing an Overall “Hazard Identification Approach”

Building on the first two steps, the Panel designed a testing scheme—a Hazard Identification Approach—for detecting chemicals that may raise the risk of breast cancer. The Hazard Identification Approach consists of prioritization criteria and a proposed toxicity testing approach. Figure 7 is a schematic representation of the recommended approach.

Prioritization: Of the tens of thousands of chemicals in use, the Panel recommends prioritizing those with highest potential for human exposure and those with preliminary indicators of hazard. While the Panel supports the goal of comprehensive testing of all synthetic chemicals, the backlog of untested chemicals necessitates making decisions about where to start. Chemicals, their metabolites and degradation products should be prioritized for testing based on the following parameters:

- **Exposure potential**--Chemicals that are most likely to raise breast cancer risk are those to which people are commonly exposed. Indicators of potential exposure include biomonitoring studies (e.g., the CDC National Biomonitoring program),⁹⁴ environmental monitoring,^{95 96} or other proxy measures of exposure, such as high production volume, tendency to bioaccumulate or persist, or widespread use in consumer products or workplaces. Exposure potential should be assessed at all points in the product lifecycle, from production through use and disposal. Priority should also be given to chemicals that will likely enter human breast tissue and milk. Exposure potential should be assessed across the full human lifecycle in order to address risks associated with different life stages,

from prenatal development through menopause.

- **Hazard indicators**-- Many chemicals with insufficient toxicity data nevertheless have one or more characteristics that could indicate a potential to raise the risk of breast cancer. These include structural similarities to other mammary gland carcinogens, indicators that a chemical or its metabolite could have endocrine activity, alter breast development, change gene expression, or create genetic mutations.

Toxicity Testing

The Hazard Identification Approach recommended by the Panel consists of three categories of biological endpoints associated with an increased risk of cancer in general, and breast cancer in particular. These categories are:

- Mechanisms associated with carcinogenesis in general, including cell cycle changes and genotoxicity;^{97 98}
- Mechanisms associated with endocrine disruption;⁹⁹ and
- Altered development and maturation of the mammary gland.¹⁰⁰

The Panel chose these categories of endpoints based on modes of action for known breast carcinogens, the scientific expertise and opinion of Panel members, literature reviews, and the availability of validated toxicity testing assays. The rationale for choosing each category of endpoints is detailed below. Two of the endpoints, endocrine disruption and mechanisms of carcinogenesis in general, can be assessed by relatively rapid *in vitro* assays and/or whole animal (*in vivo*) studies. The last category, altered mammary gland development, currently can only be assessed by *in vivo* studies.

The Panel chose not to recommend specific assays, since test methods are changing rapidly. However, if this approach were implemented today, some examples of currently available assays are given in Figure 7.

Chemical Prioritization

Chemicals, their metabolites and degradation products, should be prioritized for testing based on the following parameters:

Hazard indicators

including structural similarities to other mammary gland carcinogens, or indicators that a chemical or its possible metabolite have endocrine activity, alter breast development or gene expression, or create genetic mutations.

Exposure potential

predicted by physical-chemical properties that indicate potential for bioaccumulation, persistence in the environment, or result in exposure to breast tissue. Also those identified by biomonitoring, environmental monitoring, or other proxy measures such as high production volume or dispersive use in consumer products or workplaces. Exposure potential should be assessed across the entire human life-cycle, and the product lifecycle from manufacturing through disposal.

Hazard Identification Approach

Rapid (in vitro) screening

Genotoxicity

Mutagenicity (e.g., Ames or equivalent)
Chromosome aberrations (e.g., OECD TG 473)
Micronuclei formation (e.g., OECD TG 487)
DNA strand breaks (e.g., COMET assay)

Cell cycle changes

Cell division (e.g., ³H thymidine proliferation assay)
Altered apoptosis (e.g., TUNNEL assay)

Endocrine disruption

Activation or inhibition of:
Estrogen-mediated transcription (e.g., E-screen)
Androgen-mediated transcription (e.g., A-screen)
Enzymes specific to synthesis or metabolism of estrogen, androgen or progesterone (e.g., aromatase activity assay)

Animal studies (in vivo): development and maturation

Genotoxicity in breast epithelial cells

Mutagenicity
Chromosome aberrations
Micronuclei formation
DNA strand breaks

Precursor changes, biomarkers and induction of mammary gland tumors

Modification of existing long-term cancer bioassays* redesigned to evaluate mammary gland endpoints, and:
include whole mounts of mammary tissue
include in utero exposures
assess effects over the whole lifespan
use an animal strain appropriate to the exposure and the endpoint

Cell cycle changes in breast epithelial cells

Cell proliferation
Decreased apoptosis

Endocrine disruption

Estrogenic activity (e.g., Uterotrophic assay)
Androgenic activity (e.g., Hershberger assay)
Developmental changes in female and male mammary gland tissue (e.g. TEB formation, ductal branching, ER and AR levels)
Reproductive changes in males and females (e.g., AGD, nipple retention, altered cyclicity, pubertal timing)
Altered circulating hormone levels (e.g. steroid or peptide hormones)

*Assessed in OECD extended one generation bioassay or the NTP enhanced Reproductive Assessment by Continuous Breeding protocol

Figure 7. Proposed criteria for prioritization and proposed “Hazard Identification Approach” for identifying potential breast carcinogens

Mechanisms associated with endocrine disruption

The rationale for recommending endocrine disruption tests is based on 1) animal models demonstrating that prenatal exposure to steroid hormones (such as androgen, estrogen, or progesterone) increases the likelihood of developing mammary gland tumors after exposure to a known carcinogen later in life;¹⁰¹ 2) evidence of associations between endogenous hormones or estrogen and progesterone-containing hormone replacement therapy and increased breast cancer risk;¹⁰² and 3) findings that increased estrogen exposure during sensitive developmental windows—whether from endogenous, exogenous, or xenobiotic sources—can increase breast cell proliferation, tissue growth, and consequently breast cancer risk.¹⁰³ The Panel concluded that chemicals with either direct or indirect estrogenic effects should be strongly suspected of increasing breast cancer risk. The Panel further concluded that chemicals that disrupt other sex steroid hormonal systems should also be considered for immediate testing.

Based on currently available toxicity testing methods, the Panel identified methods of *in vitro* screening for estrogenic and androgenic activity, as well as for steroid synthesis (steroidogenesis). Estrogen- and androgen-mediated transcriptional assays and a steroidogenesis assay have been validated for use in the U.S. EPA Endocrine Disruptor Screening Program (EDSP),¹⁰⁴ although the Panel felt that some of these assays are not suitable for evaluating breast cancer risk. For example, the steroidogenesis assays which have been validated for the EDSP need to be adapted to use isoforms of enzymes specific to breast tissue. Furthermore, the use of an animal strain appropriate for the exposure and endpoint, feed, and housing is critical when conducting whole animal tests and should be considered when designing an assay.

Although an assay could presumably be readily developed to screen chemicals for progesterone activity, the Panel could not identify any rapid

screening methods currently available for this endpoint, and few chemicals have yet to be identified as progesterone disruptors.¹⁰⁵

Mechanisms associated with carcinogenesis in general

The rationale for recommending this set of endpoints is based on the general acceptance that they have been traditionally associated with the development of cancer. Furthermore, these endpoints are also routinely evaluated in pharmaceutical and pesticide testing and a number of validated assays are currently being used. Finally, a number of identified mammary carcinogens have been found to be positive as genotoxic agents.¹⁰⁶ The categories of endpoints identified by the panel as being important to evaluate in toxicity testing were a) cell cycle changes, and b) genotoxicity.

Cell cycle changes include processes such as increased cell replication or decreased apoptosis (programmed cell death). Both endpoints are widely recognized as markers of increased cellularity, which could lead to limitless cell replication, a hallmark of cancer.

Genotoxicity includes mutagenicity and clastogenicity. Mutagens (e.g. radiation) increase the rate of mutations, and clastogens (e.g. benzene) damage DNA structure. Most identified chemical mutagens act indirectly, causing damage such as DNA adducts or DNA strand breaks rather than changing the primary nucleotide sequence. In these cases, mutation occurs via a complex system of signaling pathways usually involving enzymatic activities and DNA replication.¹⁰⁷ Epoxide chemicals, or substances that are metabolized to epoxides, can form DNA adducts which result in mutations. Several of these chemicals induce mammary tumors in laboratory animals, and have been linked to breast cancer in humans.¹⁰⁸ Impaired DNA repair has also been associated with development of breast cancer.¹¹⁰

At least half of the mammary carcinogens identified by the NTP have been found to be

mutagenic in *Salmonella* and more are positive in additional genotoxic assays.¹¹¹

Standard genotoxicity test batteries have been adopted by the International Congress for Harmonization (ICH) Guidelines, the gold standard for assessing compounds used in clinical trials of human subjects.¹¹² These tests have been incorporated into the U.S. Food and Drug Administration (FDA) guidelines. Testing for mutagenicity and clastogenicity using four different endpoints is now necessary for a new drug approval or food ingredient notifications. Current batteries exist for assessing genotoxicity and mutagenicity and are employed not only by the pharmaceutical industry, but also the U.S. EPA, and the European Union when assessing a chemical's toxicity. These methods are continually being improved and adopted as research evolves.

Based on current scientific knowledge and available testing methods, the Panel identified four endpoints indicative of genotoxicity (mutagenicity, chromosomal aberrations, DNA strand breaks, and micronuclei formation),¹¹³ and two endpoints indicative of cell cycle changes (decreased apoptosis and increased cell division) in the Hazard Identification Approach (*Figure 7*). These endpoints should be assessed when screening chemicals for potential carcinogenicity in general, and breast carcinogenicity in particular. In accordance with current regulatory guidelines that rely on standard genotoxicity tests, a positive result in any of the tests could provide strong indication of potential carcinogenicity.

The Panel did not want to specify assays for evaluating the four genotoxicity endpoints (e.g., Ames test) or cell cycle changes (e.g., ³H-thymidine proliferation assay), since the rapid evolution of test methods will make more efficient and less expensive assays available in the near term. This includes the high-throughput screens for genotoxicity that are currently undergoing development and validation by the U.S. EPA ToxCast program.¹¹⁴

Altered development and maturation of the mammary gland

Exposure of mammary tissue to certain chemical substances—particularly hormones or endocrine disrupting chemicals—during critical periods of development has been shown to alter mammary gland development, and increase susceptibility to future carcinogen exposure (reviewed in the Background section). A recent multidisciplinary workshop of over 70 experts determined by majority opinion that changes in mammary gland development “*could be interpreted as adverse effects because they represent alterations in growth and development and may reflect altered susceptibility to carcinogenesis and/or lead to lactation effects.*”¹¹⁵

Based on this evidence, the Panel identified *in vivo* tests that could be used to detect chemicals that increase the risk of breast cancer through the process of altered breast development. These tests include rodent assays using mammary gland whole mounts to investigate tissue-level endpoints such as altered ductal branching, extent of growth, or the relative proportions of terminal end buds, lobules, and terminal ducts. The Panel also identified morphological changes in other reproductive tissues that could serve as markers of altered development, such as retained nipples in males, shortened ano-genital distance (AGD) or changes in estrous or menstrual cyclicity.

Histological differences between the rodent and human breast—and the need for more efficient tests that reduce the use of experimental animals—underscore the need for continued research efforts to develop better tissue models, with rodent models being useful in the interim. Additionally, to date all known human breast carcinogens have been shown to induce mammary tumors in animals. However, most chemicals which have been found to induce tumors in animals have not been adequately studied in humans.

Implementation of the Hazard Identification Approach

Though the Panel did not specify assays or protocols to be used in evaluating each of the endpoints in the three categories of endpoints, the Panel did emphasize that it is important that chemical testing begin now, rather than waiting for the development of more tests or implementation of high-throughput technology. With this in mind, the Panel gave examples of currently available assays that could be used to assess each of the endpoints. These are shown in Figure 7. All of the assays in the *in vitro* section of the Hazard Identification Approach have been validated, and protocols are available in the published literature. Some of the assays in the *in vivo* section of the Hazard Identification Approach have been validated, such as the Hershberger and Uterotrophic assays, and others, such as evaluation of mammary gland whole mounts are widely used in research laboratories and could be validated in the short-term.

Balancing the use of in vivo tests

For some users of the Hazard Identification Approach, results of the cell-based (*in vitro*) assays described above will provide enough information for decision making. For others, it will not provide enough information to support decision-making on a chemical's use and hazard potential. When more information is required about the potential of a chemical to increase the risk of breast cancer, whole animal (*in vivo*) testing may be necessary. In particular, chemicals that exert toxic effects only after metabolic activation could be missed by cell-based (*in vitro*) testing methods in current use.

The Panel identified additional endpoints related to endocrine disruption and general carcinogenesis that could be readily evaluated in animal models. For endocrine disruption this includes further evaluation of estrogen and androgen activity through currently validated assays that measure reproductive organ weights (e.g. uterotrophic assay for estrogens and Hershberger assays for androgens), and measures of circulating serum hormone levels. For general carcinogenesis endpoints, this includes examination of breast epithelial cells for the previously described four indicators of genotoxicity (mutagenicity, chromosomal aberrations, DNA strand breaks, and micronuclei formation); and two indicators of cell cycle changes (decreased apoptosis and increased cell division).

In considering the use of animal models, the Panel identified the importance of testing chemicals for their potential effects on breast tissue during critical periods of development, particularly the gestational, neonatal, and pre-pubertal periods. This requires conducting bioassays that include long term follow up to evaluate the effect of these exposures on mammary gland development and function (e.g. lactation capacity). Furthermore, in all animal based tests, selection of animal strains must be appropriate to the exposure and endpoint of concern. A number of rodent strains have been classified by the NTP by their sensitivity to hormonal mammary carcinogens. These include the Sprague-Dawley, Fisher 344, Wistar Furth, and Wistar Han which are all considered susceptible to hormonal mammary carcinogens. However, the Wistar Kyoto, Copenhagen and genetically intact mice (mmtv negative) are known to be resistant.¹¹⁶

V. Discussion

The BCCP project piloted a process for recommending a set of toxicity tests (the Hazard Identification Approach) designed to detect chemicals that could increase breast cancer risk, attempting to answer the following questions:

- What chemical hazard information would help consumers identify substances to avoid, assist manufacturers in developing safer chemicals, and guide policy makers in determining which chemicals merit regulation?
- Are there widely-available methods that can be applied efficiently, reliably and cost-effectively for identifying chemicals that may raise the risk of breast cancer, and can these “standard” tests be converted to a high-throughput method?
- What approaches are offered by newly-emerging science, and what are the most pressing data gaps?

Answering these questions could help address needs identified by many expert groups, including the most recent President’s Cancer Panel report that highlighted—for the first time in its nearly 40-year history—the substantial impact of environmental exposures on increased cancer risks. The 2008-2009 annual report found that “the true burden of environmentally induced cancers has been grossly underestimated” and recommended significant changes to better protect people from cancer-causing chemicals, including action at federal, industrial, scientific, local, and individual levels.¹¹⁷ The report highlights many environmental exposures that have been linked to breast cancer, including bisphenol A, flame retardants, solvents, and radiation. The Panel’s recommendations include:

- Stronger oversight of environmental contaminants with a shift in the burden of proof for chemical safety to chemical manufacturers;

- Full disclosure of information about environmental cancer risks, including to workers and communities;
- Support for “green chemistry” and the development of safer chemicals; and
- Special consideration for vulnerable populations - including fetuses, infants, children, workers and people living in toxic “hotspots” with high levels of contamination.

These goals are consistent with recent chemicals policy initiatives in the EU and in the U.S. Accomplishing these goals, however, will require the generation and disclosure of significant information on the potential hazards of chemicals found in the environment, or used in the workplace and in consumer products. This includes identifying chemicals that may increase the risk of human disease, such as breast cancer, as well as identifying safer substitutes that can serve to replace hazardous chemicals.

The information generated by the proposed Hazard Identification Approach will be useful in implementing new chemicals policies, as well as for assessing hazards within the industrial chain of commerce and consumer product lifecycle:

- Government could use the results from the proposed testing strategy to identify chemicals that present unacceptable risks to human health and the environment;
- Chemical producers and product manufacturers could follow the approach for screening new and existing products for potential carcinogens and for incorporating principles of green chemistry as explicit design criteria; and
- Consumers might avoid products containing chemicals that have tested positive on some of these tests, or request their elimination from products.

Finally, because the biological processes identified by the Panel are relevant to many diseases, toxicity tests to assess chemicals that

affect breast development, endocrine activity, DNA, cell cycle control and other events associated with general mechanisms of carcinogenicity are also likely to identify chemicals associated with outcomes such as teratogenesis, infertility, spontaneous abortion, and cancers in other organ systems.¹¹⁸⁻¹²²

Therefore, a strategy designed to screen chemicals for potential breast carcinogenicity may have wide applicability to many disease processes in humans.

A. Recommendations: Steps to take now

The Panel recommends that chemicals used in industrial processes or found in the environment, consumer products, or workplaces be tested for their potential to increase the risk of breast cancer using the proposed Hazard Identification Approach. As presented in the Results section, testing should routinely evaluate the following endpoints relevant to breast cancer:

- Mechanisms associated with carcinogenesis in general, including cell cycle changes and genotoxicity;
- Endocrine disruption (estrogenicity and other hormonal effects) and ;
- Altered mammary gland development or maturation.

Chemicals that exhibit any of these effects should be recognized as potential contributors to the risk of breast cancer. A chemical does not have to test positive in all three categories to act as a breast carcinogen, and a null finding from one test should not be interpreted to mean a chemical is safe until it has been evaluated by the other tests. Likewise, a chemical that tests negative in the *in vitro* screens should be re-screened when assays are developed to detect newly identified endpoints of concern.

Where methods exist, toxicity testing should be conducted in rapid cell-based assays that can screen large numbers of chemicals quickly and inexpensively. For a subset of chemicals for which additional data are needed, animal-based tests more specific to mammary gland

endpoints could be conducted. These animal tests should also be conducted on chemicals suspected of acting by mechanisms that would not be detected by *in vitro* assays, such as chemicals with active metabolites.

In evaluating the potential of a chemical to raise the risk of breast cancer, the Panel recommends that toxicity tests be designed and conducted to account for *timing of exposure* and *underlying susceptibility factors*.

As a result, toxicity tests should:

- Assess the impact of chemical exposure during a variety of life stages, including gestation, puberty, pregnancy, and post-menopause;
- Account for increased susceptibility due to genetic variation (e.g., BRCA1 and BRCA2, or polymorphisms that affect metabolism of xenobiotics),¹²³ underlying disease, vulnerable life stage, or exposure to other chemicals and environmental stressors; and
- Account for racial/ethnic disparities in disease.

These goals can be accomplished through appropriate design of *in vitro*, animal-based and epidemiologic studies and by incorporating new techniques as they become available.

B. Recommendations: Research needs

The primary aim of the BCCP project was to develop recommendations for chemical testing based on current knowledge of alterations in biological processes associated with breast cancer and existing toxicity testing methods for assessing those endpoints. The Hazard Identification Approach developed by the Panel reflects only currently available methods, and the Panel recognized the need for more research to improve these methods.

To help direct future research, the Panel analyzed gaps in two areas described below:

1) Characterizing alterations in biological processes associated with breast cancer susceptibility, disease causation and progression; and

2) Improving toxicity testing methods.

1. Characterizing Alterations in Biological Processes

The Panel's proposed Hazard Identification Approach is limited to assays that evaluate the endpoints within biological processes that existing evidence strongly links to breast cancer. The ability to identify potential breast carcinogens would be improved by research that better characterizes the biological processes that, when altered, increase the development or progression of, or susceptibility to breast cancer. This includes identifying early events in a biological pathway—such as altered development of the mammary gland—that occur well “upstream” of tumor formation.

In the near term, the Panel recommends better characterization of:

- factors that modulate hormonal activity, including breast-specific hormone synthesis (e.g., aromatase activity) and genetic polymorphisms that alter hormone metabolism; and
- the role of epigenetic changes in breast carcinogenesis.

Better characterization of the relationships between chemical exposure, biological alterations, and the ultimate progression to breast cancer will improve the predictive value of any observed changes.

2. Improving Toxicity Testing Methods

The Panel identified three research needs for improving testing of potential breast carcinogens: a) adapting existing assays to improve their relevance to breast cancer; b) developing new assays to evaluate biological processes important in breast cancer—both known and novel; and c) developing higher throughput screening methods and enhancing computational toxicology.

- a) Current toxicity testing methods should be modified to more specifically address mechanisms relevant to breast cancer.

Although the recommended Hazard Identification Approach draws on commonly used testing methods, some existing methods do not currently evaluate endpoints specific to the mammary gland but would be well-suited to doing so. For example, the aromatase activity assay which has been validated for use in EPA's Endocrine Disruptor Screening Program utilizes an isoform of aromatase found in the adrenal gland that varies slightly in sequence and tissue-specific regulation from the aromatase isoform found in both normal breast tissue and breast tumors. Some of the assays designated in the Hazard Identification Approach will require additional refinement and validation for use in industry and regulatory settings.

- b) New toxicity testing methods should be developed and validated to detect events in biological processes that are likely to alter breast cancer risk but for which current test methods are inadequate. These include:

- progesterone receptor binding and transcriptional activation,
- protein hormone activity (e.g. growth factors or prolactin),
- DNA enzyme repair mechanisms, and
- mechanisms associated with carcinogenesis in general, such as immune modulation, oxidative stress, and cell cycle changes that lead to increased cell proliferation or decreased apoptosis.

For example, protein hormones (e.g. growth factors such as IGF) and prolactin are suspected of being important modulators of breast cancer risk,^{124 125} indicating a need to better understand how chemicals may alter these pathways and a need for developing toxicity testing methods to evaluate these endpoints.

As additional associations between biological changes and breast cancer are better characterized, new screening assays should be developed, their predictive power should be characterized, and they should be validated for use in industry and regulatory settings.

c) High-throughput screens and computational models should be expanded. As many reports have established, standard toxicity testing approaches that rely on lengthy *in vivo* assays cannot feasibly screen all existing substances for their contribution to breast cancer risk.¹²⁶⁻¹²⁸

New screening methods are needed to:

- Reduce reliance on time-consuming and costly animal-intensive assays,
- Increase the ability to rapidly screen a large number of chemicals to identify potentially hazardous chemicals and facilitate action, or
- Identify chemicals that merit more in-depth studies in experimental animal models.

This recommendation follows the lead of major new initiatives in chemical hazard evaluation that seek to shift emphasis from decades-old whole animal testing protocols to more efficient *in vitro* mechanism-based chemical screening.^{129 130} For example, U.S. EPA is investigating the predictive power of a series of 400 *in vitro* screening tests that have been applied to 300 chemicals with well-characterized toxicity profiles in whole animal tests.¹³¹ The U.S. National Toxicology Program is also developing *in vitro* screening tests to be applied to a set of 10,000 chemicals.¹³²

Some high-throughput screening techniques currently used in pharmaceutical research to identify the pharmacokinetics and molecular targets of potential new drugs could be applied in toxicity testing to predict hazard based on biological activity.^{133 134} In addition, embryonic stem cell lines hold promise as research tools to predict the effects of environmental chemical exposures on human health.^{135 136}

Finally, computational models that predict hazard or exposure potential based on inherent

The information generated by the proposed Hazard Identification Approach will be useful in implementing new chemicals policies, as well as for assessing hazards within the industrial chain of commerce and consumer product lifecycle.

chemical properties and structural similarities (QSAR) should be improved and used to prioritize chemicals for further testing. For example, some chemical structures are associated with distinct types of mutations that raise cancer risk.

One such *in silico* method was investigated during the BCCP project using existing databases to identify several key molecular targets and activating pathways for chemical compounds associated with mammary tumors in animals. An evaluation was conducted to determine whether these targets and pathways could serve as triggers for structural alerts, with results indicating that QSAR models could assist in guiding chemical screening for agents likely to be involved in breast cancer development and progression.¹³⁷

While the Panel recommends the use of computational models for generating hypotheses on which to base further investigation of a chemical's biological effects, the absence of indicators of hazard in such models should not constitute proof of safety.

Addressing these research needs will require significant interdisciplinary efforts to link current knowledge of breast cancer etiology with the design and implementation of chemical toxicity testing.

VII. Conclusion

Chemical toxicity testing—and the public policies that establish requirements for such testing—are two critical tools in breast cancer prevention, since they provide a practical basis for reducing exposure to chemicals that may increase breast cancer risk.^{138 139} The Hazard Identification Approach developed by the Panel could guide the development of toxicity testing specific to breast cancer. Information generated by implementing the Hazard Identification Approach could a) increase the relevance of chemical assessments for public health; b) provide a scientific basis for identifying chemicals that may increase breast cancer risk; and c) generate data to support use of less toxic alternatives.

More comprehensive and efficient detection of chemicals linked to breast cancer will require both ongoing research into the biological basis of breast cancer and the development of new toxicity testing methods, particularly the development of *in vitro* chemical screening techniques and high-throughput methods.

Meanwhile, it is essential that practical approaches to identifying potential breast carcinogens are implemented now to begin addressing the backlog of untested chemicals and informing the development of new chemicals policies. These approaches will by necessity depend on currently available methods (e.g. tests for estrogen-like effects, or genotoxicity), but they should also include tests used in other settings that can be adapted to include endpoints relevant to breast cancer. For

Chemical toxicity testing—and the public policies that establish requirements for such testing—are two critical tools in breast cancer prevention.

example, existing toxicity testing guidelines such as the OECD extended-one generation studies could be readily modified to evaluate changes to mammary gland development after chemical exposure.¹⁴⁰

The same process piloted by the BCCP project could be used to develop approaches for identifying chemicals that contribute to the risk of other diseases, especially diseases for which mechanisms of action are fairly well understood (e.g. thyroid disruption and neurodevelopmental delays). In practice, regulatory agencies as well as chemical producers and users will need information on chemicals linked to many diseases (beyond breast cancer) in order to assess hazardous chemicals and their safer alternatives. The ultimate aim is to integrate the testing strategies used for a variety of disease endpoints into a comprehensive approach to chemical hazard identification.

The proposed Hazard Identification Approach recommended by the Panel could be applied to many chemicals, prioritizing a subset for further testing, identifying hazards and their safer alternatives, and generating toxicity information useful for consumers, product manufacturers, workers, chemical producers, and policy makers.

Appendix 1: BCCP Participants

Project Leaders:

Sarah Janssen, MD, PhD, MPH (Co-Director)

Senior Scientist, Natural Resources Defense Council
Assistant Clinical Professor
Occupational & Environmental Medicine
UC San Francisco, CA

Megan Schwarzman, MD, MPH (PI and Co-Director)

Center for Occupational and Environmental Health,
UC Berkeley; and Clinical Instructor, Family and
Community Medicine
UC San Francisco, CA

Panel Members:

Susan Braun, MA

Executive Director, Commonweal
Bolinas, CA

Vincent James Coglian, PhD

Head, IARC Monographs Programme
International Agency for Research on Cancer
World Health Organization
Lyon, France

***Shanaz Dairkee, PhD**

California Pacific Medical Center Research Institute
San Francisco, CA

Suzanne Fenton, PhD

Reproductive Endocrinologist
Cellular and Molecular Pathology Branch
National Toxicology Program, NIEHS
Durham, NC

William H. Goodson, III, MD

Senior Clinical Research Scientist
California Pacific Medical Center Research Institute
San Francisco, CA

***Joe Guth, PhD, JD**

Legal Director
Science and Environmental Health Network
Albany, CA

Dale Johnson, PharmD, PhD

Adjunct Professor
Nutritional Science & Toxicology
College of Natural Resources
UC Berkeley, CA
President & CEO, Emiliem

Jean Latimer, PhD

UPCI Hillman Cancer Center
School of Medicine
University of Pittsburgh, PA

Ron Melnick, PhD

Retired Associate
Environmental Toxicology Program
National Institute of Environmental Health Sciences
Research Triangle Park, NC

Rachel Morello-Frosch, PhD, MPH

Associate Professor
Environmental Science, Policy and Management
Division of Society and Environment
College of Natural Resources
UC Berkeley, CA

Ruthann A. Rudel, MS

Director of Research
Senior Scientist, Toxicology and Risk Assessment
Silent Spring Institute
Newton, MA

***Gina Solomon, MD, MPH**

Senior Scientist, Natural Resources Defense Council
Associate Clinical Professor
Occupational and Environmental Medicine and
Internal Medicine
UC San Francisco, CA

Carlos Sonnenschein, MD

Professor
Department of Anatomy and Cellular Biology
Tufts University School of Medicine
Boston, MA

***Lauren Zeise, PhD**

Chief, Reproductive and Cancer Hazard Assessment
Office of Environmental Health Hazard Assessment
California EPA, Oakland, CA

Funding Agency:

California Breast Cancer Research Program

UC Office of the President, Oakland, CA

Marion H.E. Kavanaugh-Lynch, MD, MPH, Director

**Catherine Thomsen, MPH, Project Lead, Special
Research Initiatives**

Administrative Staff: Suzanne Llewellyn, MEd

** Member of the Core Advisory Panel Group*

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