Mini Review

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Testing the low dose mixtures hypothesis from the Halifax project

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Abstract: In 2013, 60 scientists, representing a larger group of 174 scientists from 26 nations, met in Halifax, Nova Scotia to consider whether – using published research – it was logical to anticipate that a mixture of chemicals, each thought to be non-carcinogenic, might act together in that mixture as a *virtual carcinogen*. The group identified 89 such chemicals, each one affecting one or more Hallmark(s) – collectively covering all Hallmarks of Cancer – confirming the possibility that a chemical mixture could induce all the Hallmarks and function as a virtual carcinogen, thereby supporting the concern that chemical safety research that does not evaluate mixtures, is incomplete. Based on these observations, the Halifax Project developed the Low-Dose Carcinogenesis Hypothesis which posits "…that low-dose exposures to [mixtures of] disruptive chemicals that are not individually carcinogenic may be capable of instigating and/or enabling carcinogenesis." Although testing all possible combinations of over 80,000 chemicals of commerce would be impractical, prudence requires designing a methodology to test whether low-dose chemical mixtures might be carcinogenic. As an initial step toward testing this hypothesis, we conducted a mini review of published empirical observations of biological exposures to chemical mixtures to assess what empirical data exists on which to base future research. We reviewed studies on chemical mixtures with the criteria that the studies reported both different concentrations of chemicals and mixtures composed of different chemicals. We found a paucity of research on this important question. The majority of studies reported hormone related processes and used chemical concentrations selected to facilitate studying how mixtures behave in experiments that were often removed from clinical relevance, i.e., chemicals were not studied at human-relevant concentrations. New research programs must be envisioned to enable study of how mixtures of small doses of chemicals affect human health, starting, when at all possible, from non-malignant specimens when studies are done *in vitro*. This research should use human relevant concentrations of chemicals, expand research beyond the historic focus on endocrine endpoints and endocrine related cancers, and specifically seek effects that arise uniquely from exposure to chemical mixtures at human-relevant concentrations.

Keywords: carcinogenesis; chemical mixtures; environment; Halifax project; low dose mixtures; xenoestrogens.

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Introduction

In 2013, 60 scientists, representing a group of 174 scientists recruited from 26 countries, met in Halifax, Nova Scotia and reached the unanimous conclusion that there is already sufficient published scientific evidence to state that a mixture of chemicals thought to be benign, in doses small enough to be considered safe, *might* work together –as a mixture – to cause cancer, i.e., to be a virtual carcinogen [1]. The results of the Halifax Project, as the meeting has come to be called, built on concern about mixtures that had been expressed previously [2, 3]. The contribution of the Halifax Project was to gather in one place a group of scientists, each one of whom was an expert in one or more of the Hallmarks of Cancer. The goal was for the group to share perspectives and collectively to envision whether and how effects on individual Hallmarks of Cancer *might* create cancer from the complementary effects of simultaneous exposure to separate chemicals, none of which are considered carcinogenic individually. In preparation for the conference, participants reviewed published literature to facilitate designing a testable hypothesis concerning a possible role of putatively safe chemicals – as mixtures – in carcinogenesis. Key background concepts were that chemicals accumulate in the body, environmental chemicals may have adverse effects, and most previous chemical safety research has evaluated individual chemicals or limited mixtures of chemicals with like structure or action. A review of the possible role of environmental chemicals in carcinogenesis *per se* was not part of the project, but it was noted that the epidemiology of cancer and environmental chemicals continues to evolve. This is especially true for breast cancer (Table 1 [5–43]) for which incidence increased significantly over four decades [44] at the same time that incidence decreased for other cancers such as colon, lung, prostate, ovary, and cervix [44].

Two years prior to the conference, the non-profit organization, Getting to Know Cancer, sent emails to roughly 40,000 scientists describing the Project and inquiring about interest in participation. From nearly 1,000 respondents, 174 scientists from 26 nations were invited and became part of one of 12 working groups (Table 2). One group was assigned for each of the 10 Hallmarks of Cancer [4], and an 11th group sought effects on the microenvironment as a whole. A 12th group reviewed each chemical that one of the other teams identified as disruptive and enabling of a cancer hallmark or hallmarks to determine whether or not that same chemical had ever been reported to demonstrate any effects either favoring or reducing carcinogenesis. Many of the scientists were recruited for their expertise in cancer biology (with an emphasis on the various Hallmarks of Cancer). Because many of the chemicals previously associated with cancer have endocrine effects, the project specifically recruited environmental toxicologists familiar with endocrine disruption to ensure that most teams had participants with a good understanding of the mechanisms involved and the effects that disruptive chemicals have on those mechanisms.

Before the meeting in Halifax, all the members of each Hallmark group reviewed existing literature to identify chemicals that met both screening criteria and action criteria. *Screening* criteria meant the chemical was in the environment and considered safe. *Action* criteria meant that the chemical affected the group's Hallmark in a way that was similar to how that Hallmark functioned or occurred in cancer. Effects were considered when they occurred either: a. in an exposure range measured in humans; b. at a dose lower than usual testing; c. at a dose below the lowest observed effect for carcinogenesis; or d. at animal blood or tissue levels similar to those found in humans. Mutagens that had broad or non-specific effects were excluded, because they would be expected *a priori* to be carcinogens. However, mutagens known to act consistently and precisely in a particular way (i.e., signature mutations), for example, in certain types of adduct formation [45] were acceptable for inclusion.

Carcinogenesis by addition of effects is conceptually the reverse of toxicology and/or cancer treatment that typically work by disruption of ongoing processes, e.g., genomic stability, endocrine homeostasis, etc. where disruption at a single site may be sufficient to derail the entire process. Carcinogenesis, in contrast, requires the presence of the effects of multiple Hallmarks, single effects of which may not be sufficient to cause cancer. For example, multiple mutations – even cancer associated mutations – may exist in a tissue and not cause cancer, as shown by multiple mutations in esophageal [46] or eyelid tissues [47] that are not malignant. This view of treatment versus carcinogenesis is supported by Rothman's metaphor of a *causal pie* [48] in which each contributing cause that is necessary for a cancer to exist is thought of as one *piece of a pie*. When all pieces are present, cancer exists. However, if one piece is removed, the pie fails because it is incomplete. This is the basis for much of cancer treatment where treatment of one or a few necessary causes is sufficient to stop the cancer. In contrast, carcinogenesis requires the accumulation of a number of complementary causes to produce cancer. Moreover, it is likely that there may be many possible causal pies for carcinogenesis (i.e., each one involving different sets of complementary causes, that are together capable of enabling carcinogenesis) [49].

The chairpersons and representatives from each working group attended the meeting in Halifax. Every project group identified one or more chemical(s) that met these screening and action criteria. In total, 89 chemicals were identified that adversely affected one or more Hallmark(s). Fifty (59%) of these chemicals had effects at low doses (It is likely others exist that were not identified in the time allowed). Having found at least one adversely acting chemical for each Hallmark, the Project concluded there was a *real possibility* that a mixture of selected chemicals could induce each and every Hallmark, and thus a mixture of supposedly safe chemicals might behave as a virtual carcinogen. The Project did not prove that such a mixture exists, but it found enough evidence to conclude that we cannot ignore this possibility. A table of chemicals favoring

Table 1: Examples of the evolving epidemiology of a sample of three environmental chemicals.

First author	Year	Setting	Study results
Diethylstilbestrol (DES)			
Clear Cell Carcinoma (CCC) of the Vagina			
We found no studies refuting carcinogenic effect of DES on CCC of the Vagina			
Studies showing carcinogenic effect of DES on CCC of the Vagina			
Herbst [5]	1971	Eight cases, each matched to four controls born within four days in same hospitals	All CCC of the vagina occurred after *in utero* exposure when mom took DES. Significantly more prior miscarriages and bleeding durng pregnancy in moms prescribed DES
Hatch [6]	1998	American cohort of DES daughters	Increased CCC of the vagina, SIR 40.7, in daughters
Verloop [7]	2010	Netherlands cohort DES daughters	Increased CCC of the vagina, SIR 24.2, in daughters
Breast Cancer (BRCA)			
Studies finding no carcinogenic effect of DES on breast cancer (BRCA)			
Brian [8]	1980	Mothers who took DES while pregnant 1940 - 1960	No increased risk of breast cancer in mothers
Hatch [6]	1998	American cohort of DES daughters	No increase in BRCA in daughters
Veerloop [7]	2010	Netherlands cohort DES daughters	No increase in BRCA in daughters
Studies finding carcinogenic effect of DES on BRCA			
Greenberg [9]	1984	Mothers who took DES while pregnant 1940–1960	Increased risk of breast cancer (RR 1.4) in mothers who had taken DES
Titus-Ernstoff [10]	2001	Mothers who took DES while pregnant 1940–1960	Increased risk of breast cancer (RR 1.27) in mothers who had taken DES
Palmer [11]	2006	American Cohort of DES daughters	Increased risk of breast cancer (RR 1.91 $\geq$ 40 years age) in daughters exposed *in utero*
Hoover [12]	2011	American Cohort of DES daughters	Increased risk of breast cancer (HR 1.82 $\geq$ 40 years age) in daughters exposed in *utero*
Tournaire [13]	2015	French cohort of DES daughters	Increased risk of breast cancer (SIR 1.17) in daughters exposed in *utero*
Troisi [14]	2019	American Cohort of DES daughters	Increased risk of breast cancer (SIR 1.17) in daughters exposed in *utero*
Dichlordiphenyltrichloroethane (DDT) and long-term or past exposure indicated by its Dichlordiphenyldichloroethylene metabolite (DDE)			
Studes showing no carcingenic effects of DDT or DDE on BRCA			
Kreiger [15]	1994	Case-control at time of BRCA diagnosis through 1990 using prospective serum obtained 1960s from Caucasian, African American, and Asian women San Francisco Bay Area	DDE levels not elevated in cases
van't Veer [16]	1997	DDE levels in needle aspirates of fat from buttocks at time of BRCA diagnosis versus controls	DDE in adipose tissue not increased in cases
Schecter [17]	1997	Hospital based case-control in Vietnam BRCA versus benign breast disease	Levels DDT and DDE not increased in cases
Hunter [18]	1997	Case-control from Nurses Health Study using blood samples obtained 1989–1990	No association DDE levels with subsequent BRCA
Helzlouser [19]	1999	Serum obtained from two cohorts, the cohorts were sampled five years apart for a heart study	No association of subsequent breast cancer with serum DDE
Dorgan [20]	1999	Case-control taken from Missouri Cancer Serum Bank specimens drawn 1977–1987 with 9.5 years follow-up	No association of subsequent BRCA with either DDT or its analogs
Wolff [21]	2000	Cases of BRCA in African American, Asian, and Caucasian compared to control of patients with benign breast disease or routine screening patients without disease	Higher DDT levels in minorities compared to Caucasians, but DDT levels did not correlate with BRCA
Laden [22]	2001	Case-control taken from Nurses Health Study; blood samples drawn 1989–1990 from women with no diagnosis of cancer; BRCA diagnosis through 1994	No association of DDE with BRCA
Brody [23]	2004	Estimate DDT exposure by dates and prevailing weather in first 24 h after DDT vector spraying on Cape Cod	Breast cancer did not relate to presumed exposure to DDT from spraying
Gatto [24]	2007	Case-control African American women in Los Angels (drawn from a larger study of five major city areas) diagnosed with BRCA 1994–1998 versus controls from random dialing	DDE higher in cases, but not after adjustment for serum lipids

Table 1: (continued)

First author	Year	Setting	Study results
Itoh [25]	2009	Japanese case-control at time of BRCA diagnosis	No increase BRCA with DDT isomers or DDE; slighly lower DDT and DDE levels for cases
White [26]	2013	Adult recall of seeing a DDT fogger truck in neighborhood in childhood growing up on Long Island	No relation of seeing a DDT fogger truck to later breast cancer
Holmes [27]	2014	Blood and urine from Alaskan Native Americans with biopsy diagnosing cancer versus controls with benign breast disease on biopsy	No increase BRCA with DDE or DDT, and slightly lower levels of both in cases versus controls
Studies with increased BRCA with DDT or DDE exposure			
Hoyer [28]	2000	Serum obtained from same cohort sampled sequentially at two times, 5 years apart	Subjects with highest quartile for DDT in the average of the two samples had 3.6 odds ratio for breast cancer. DDT, but not DDE, declined in both groups over time.
Romieu [29]	2000	Cases diagnosed with BRCA in Mexico City 1990–1995 versus random sample city population	DDE (but not DDT) higher in cases; OR 2.2 highest versus lowest quintile [DDE higher in older women and lower with longer lactation]
Charlier [30]	2003	Case-control in Belgium using blood samples obtained at time of diagnosis versus controls having routine GYN care	More DDT in cases, and OR 5.36 for DDT at "limit of quantification"
Cohn [31)	2007	Case-control drawn from serum of mothers who had been pregnant and donated maternal blood for Child Health and Development Study, Oakland, CA 1959 to 1967 and subsequently develop BRCA up to age 50 in 1998.	OR 3.7 for BRCA for highest quartile DDT versus lowest quartile for mothers who had been younger, i.e., <14 years age, in 1945 the year wide use of DDT began.- There was no relation for women who had been older, i.e., 14 years or older, in 1945.
Tang [32]	2014	Case-control using DDE in blood at time of diagnosis versus controls.	Serum DDE higher in cases.
Cohn [33]	2015	Case-control drawn from daughters of women who donated maternal blood for Child Health and Development Study, Oakland, CA 1959–1967, with cases through age 52	OR 3.7 for highest quartile *in utero* exposure to DDT of daughters versus lowest exposure; also OR 2.1 for a Her2 over expressing cancer
Wielsoe [34]	2017	Serum from Greenland Inuit BRCA cases recruited in two separe groups, 2000–2003 and 2011–2014, versus controls	Total organochlorine pesticides (OCPs) and DDE higher in cases ($p < 0.001$); however, only trend for significant OR for DDE ($p = 0.002$) and not for DDT ($p = 0.074$). Overall, DDT and total OCPs lower in the second time interval, i.e., 2011–2014.
Chang [35]	2018	DDT exposure estimated by residence in regions of Taiwan sprayed with DDT 1953–1957, linking place of residence for women >5 years old at spraying to women <5 years old at spraying	Women exposed to DDT spray before age 5 had increased breast cancer by age 50–54 and the increase was higher with exposure to more sprayings
Kaur [36]	2019	Case-control comparison serum levels of DDT in India	Serum DDT higher in cases
Phthalates and Dibutyl Phthalate (DBP)			
Studies with mixed or no carcinogenic effects			
Lopez-Carrillo [37]	2010	Case-control of urine phthalate levels at time of BRCA diagnosis	All phthalates including DBP lower in cases than controls, except MEHP higher in cases
Villeneuve [38]	2011	Case-control using estimated exposure to phthalates through occupation for male breast cancer cases in Europe; note that many controls had colon cancer that was thought not to relate to chemical exposures	No association of male BRCA with working in occupation involving high phthalate exposure
Holmes [27]	2014	Case-control using blood and urine from Alaskan Native American cases with biopsy showing BRCA versus controls with benign breast disease on biopsy	MEHP higher in controls (OR 2.43 in multivariate analysis)
Parada [39]	2018	Spot urine from Long Island cases, but specimens were collected up to 3 months after diagnosis versus controls	No association of increased BRCA with any phthalate and possible inverse relation to two phthalates
Studies supporting further assessment for possible carcinogenic effects of dibutyl phthalate			
Carran [40]	2012	Case-control of men exposed to DBP through clothing application during military Service	DBP exposure linked to increased BRCA in daughters and male genital defects in sons
Reeves [41]	2019	Case-control study using urine samples within Women's Health Initiative	Overall no relation of phthalates to BRCA, except OR 9.96 for highest versus lowest quartile DBP for diagnosis of BRCA within 3 years of the biomarker measurement

Table 1: (continued)

First author	Year	Setting	Study results
Ahern [42]	2019	BRCA incidence in relation to DBP and other phthalates in prescripton medications prescribed and redeemed repeatedly over 10-year period as measure of medication use (phthalate content known from pill composition in the Danish Medicines Agency)	Women with highest ingestion of DBP through prescription medicines – over 10 years – had increased BRCA, HR 1.9. No relation to other phthalates
Enis [43]	2019	Colon cancer incidence in relation to ortho phthalates (DEP, DBP) and other phthalates in prescription medications prescribed and redeemed repeatedly over 10-year period (phthalate content from pill composition in the Danish Medicines Agency)	Decreased colon cancer with highest exposure to total ortho phthalates (OR 0.89) or DEP (OR 0.88) but not DBP. However, if omit cases who filled one or more NSAID prescription, observed increased colon cancer with highest *ortho*-phthalate exposure (OR 1.26)

carcinogenesis for each Hallmark, along with extensive references, was published in a special, open access issue of *Carcinogenesis* [1]. Based on this information, the Halifax Project proposed The Low-Dose Carcinogenesis Hypothesis "…that low-dose exposures to [mixtures of] disruptive chemicals that are not individually carcinogenic may be capable of instigating and/or enabling carcinogenesis." All 174 scientists in the 12 working groups signed on to this hypothesis [1].

After the publication of the results of the Halifax Project, we considered how best to address the daunting permutations necessary to assess even a sample of the mixtures possible from the over 80,000 known common chemicals of commerce [50]. We concluded that the first step was to tabulate published empirical observations of effects of chemical mixtures in order to learn from what has been observed heretofore. The result of that mini-review is the focus of this paper.

Table 2: The Working Groups of the Halifax Project, based on the Hallmarks of Cancer [4].

Original hallmarks of cancer
Cells sustaining proliferative signaling
Resisting cell death
Inducing angiogenesis
Enabling replicative immortality
Activating invasion and metastasis
Evading growth suppressors
Enabling characteristics hallmarks
Genome instability and mutation
Tumor-promoting inflammation
Emerging characteristics hallmarks
Deregulating cellular energetics
Avoid immune destruction
Groups unique to the Halifax project
Microenvironment as a whole
Review chemicals proposed by working groups to identify any with previously known anti-cancer effects

Previous mixtures research

The first step toward testing the low-dose mixtures hypothesis proposed by the Halifax Project is to survey the existing research with a focus on the effects of chemical mixtures. PubMed was searched using combinations of terms for *endpoints,* e.g., carcinogenesis, cancer, cell proliferation, apoptosis, angiogenesis; *models*, e.g., cell lines (including commonly used cell lines MCF7 and T47D by name), cells (which identified additional studies with fresh cells), zebra fish, mice, rats, etc.; and *the agents we were seeking*, e.g., chemicals, chemical mixtures, environmental chemicals, as well as common groups of chemicals such as parabens, phthalates, bisphenol-A, and endocrine disruptors. We are unaware of a previous attempt to tabulate mixtures research in this way.

Abstracts were reviewed and publications of interest were selected: 1 if they included observations of new experiments as opposed to reviews or discussions of previous work; 2 if the chemicals were tested as mixtures (papers that compared results from several chemicals, but tested them individually, rather than as mixtures, were not included); 3. if a fixed-ratio mixture was tested at different dilutions, it was included only if there was a comparison group with individual chemicals, a different mix of chemicals, or different proportions. This was not a formal systematic review. However, additional references were pursued using the above terms until it was realized that the same references were coming up in different searches.

The author, date, model system, endpoints, chemicals tested, exposure levels of chemicals, and a brief statement of results were collected for 58 separate studies ([51–75], [76–111]; Table 3). We did not tabulate the authors' conclusions about whether the effects of mixtures were partially additive, additive, or synergistic; but if the authors reported antagonism between chemicals, that was noted.

Several observations become clear from this survey:

Table 3: Existing research on effects of chemicals as mixtures.

	First author	Year	Model	Endpoints	Chemicals tested	How mixed	Observed results[a]
1	Bliss [51]	1939	Flies	Mortality	Pyrethrum, kerosene, barium fluorosilicate, rotenone, dinitro-cyclohexylphenone	Binary mixtures	Additive effects on mortality
2	Fukushima [52]	1991	Rodent	Tumors on necropsy	Hepatocarcinogen mixture (AAF, DMN, DAB, phenobarb, thioacetamide); nitroso compound mixture (BBN, DBN, EHEN, MNNG, PNU); antioxidant mixture	Binary and ternary mixtures of the groups with and without DMD priming	More hepatic nodules and hepatic cancers from both mixes, both with and without DMD priming; less when add in antioxidant mixture
3	Kang [53]	1996	Cells – reduction mammoplasty	Carboxyfluorescein dye transfer across Gap Junctions and Cx43	Low doses DDT, HCB, Dieldrin and other PCBs and PDDs	Binary mixtures	Additive decreased dye transfer and decreased Cx43 with both DDT + HCB and Dieldrin + HCB
4	Arcaro [54]	1997	Cells – MCF7	Growth cell foci	E2; 2,4,6TCB; 2,3,4,5TCB Dieldrin, endosulfan	With E2 and binary mixtures	2,4,6TCB additive with low E2; but no additive effects of binary TCB mixtures
5	Soto [55]	1997	Cells – MCF7	Proliferation by nuclear staining	BBP, BPA, DDE, HCB	Ternary and quaternary mixtures	Synergistic increase effects for both ternary and the quaternary mixtures
6	Payne [56]	2000	Yeast with human ER	ER reporter gene fluorescence	DDT, genestin,4-n- OP, NP	Ternary and quaternary mixtures	Additive effects both kinds of mixtures
7	Payne [57]	2001	Cells – MCF7	Proliferation by count of stained cells	*o,p′*-DDT, *p,p′*- DDE, (β-HCH, *p,p′*-DDT	Single and as quaternary mixture with 1:1:1:1 equimolar ratio or the 1:10:5:4 ratio (respectively) found in human blood	Additive effects of mixture both in equimolar and human serum ratios. Mixture effect exceeded highest single component. Effect from equimolar ratio occurred at levels below the individual NOECs.
8	Charles [58]	2002	Cells – MCF7 with ER reporter gene	ER reporter gene	MXC, DDT, Diel, BaP, CHRY, BENZ, E2, Gen	Mix A: MXC, DDT, Diel. Mix B: BaP, BENZ, CHRY. Mix C: E2, GEN, DDT	Mix A: MCX and DDT additive, little Diel effect; Mix B: most response to BaP, with some from BENZ and little from CHRY; Mix C: E2 and GEN additive, DDT antagonistic
9	Silva [59]	2002	Yeast with human ER	ER reporter gene fluorescence	2,3,4,5 TCB, DBP, DCBP, CBP, gen, DHBP, BHP, resorc, at half of EC01	Individually and mixture of the eight XEs	Mixture effect exceeded simple addition of effects
10	Rajapakse [60]	2002	Yeast with human ER reporter gene	ER reporter gene fluorescence	E2 and 2,3,4,5 TCB, DBP, DCBP, CBP, GEN, DHBP, BHP, RESORC,2,4,6 TCB, PS each at half of its EC01	E2 with and without mix of 11 XEs	Mixture additive above E2 alone
11	Bae [61]	2002	Cells – human keratinocytes	cDNA	As++ , Cd++ , Cr++ , Pb++, MNNG (maximum effect control)	As++ and mixture As++, Cd++, Cd++, PB++	Only compared As++ to mix so don’t know individual cDNA for Cd++, Cr++, or Pb++: 7 genes induced only in mix, whereas 46 genes suppressed only in mix
12	You [62]	2002	Rodent	Mammary gland morphology, PCNA IHC, serum prolactin	GEN, MXC, MIX	Pregnant dams fed test chemicals until PND 22	With mix, more alveolar and lobular growth (not seen with GEN or MXC alone) and n.s. trend to increased PCNA and prolactin

Table 3: (continued)

First author	Year	Model	Endpoints	Chemicals tested	How mixed	Observed results[a]
13 Mumtaz [63]	2002	Cells – Hela (human ovary carcinoma). HepG2 (human hepatoma)	HeLa: estrogen- responsive chloramphenico l acetyl-transferase reporter vector ERE. HepG2: CAT-TOX L commercial assay for stress promoters	DDT, DDD, DDE Ald, Die, end, As++ , Cd++, Cr(VI), Cr(III), Pb	Insecticides individually and as binary mixtures. Metals individually and as mix Cd++, Cr(III) and Pb++	No estrogenic effects insecticides individually or in binary mixures. Metal mixture had 5-fold promoter response for hMTHA, 4-fold for XRE, and lesser responses for other promoters
14 Tinwell [64]	2004	Rodent	Uterus weight	BPA, GEN, NP, EE2, MXC, DES, with E2 as control	Binary BPA and GEN; mix of all six	Binary; BPA and Gen, presence of BPA blunted effect of increasing Gen. Six chemicals; at concentrations too low to have individual effects were uterorophic as a mixture.
15 You [65]	2004	Rodent	PND90 mammary gland gene arrays	Gen, MXC	Dams fed Gen, MXC, or Mix from breeding through weaning, followed by same for pups through PND90	83 genes uniquely up regulated, e.g., lipid metabolism, and 42 genes uniquely down regulated, e.g., interleukins and casein kinases, with mix. "…not equivalent to addition of the effects associated with GEN or MXC alone."
16 Crofton [66]	2005	Rodent	T4-thyroxine	Environmental concentrations TCDD, PCDD, TCDF, PCDF(2), OCDF, PCB (12)	Single mixture of 18 EDCs	Mixture suppressed T4 50% relative to control
17 van Meeuwen [67]	2007	Cells – MCF7	proliferation by MTT reduction	OP, MXC, DBP, NP, BP, HCH (and phytochemicals not listed)	three mixtures: OP + MXC + DBP; NP + BPA + HCH; all six	Mixtures additive effects on proliferation
18 Hass [68]	2007	Rodent	Anal genital distance, nipple retention in male	Androgen receptor antagonists: VZ, FLUT, PRO	Mix of three *versus* control	Mixture more decreased anal genital distance and more retained nipples in males
19 Charles [69]	2007	Cells – MCF7 transfected with ER reporter gene; rodent	Reporter gene fluorescence; uterus weight	HCH, DPN, DDT, MXC, OP, BPA and NOEL and fractions fixed combination of phyoestrogens gen:dai	MCF7: one mix at NOEL and fractions below. Uterus:gavage same mix at LOEL	MCF7 luminensence: XEs add to gen:dai. Uterine weight: XEs add to gen:dai but less than additive
20 Howdeshell [70]	2008	Rodent	T content; T synthesis by cultured GD 18 fetal testes	BBP, DBP, DEHP, DiBP, DPP	Chemicals by gavage to GD8-18 dams in a 3:3:3:3:1 ratio respectively	No effect T in testes. DPP 3x more potent depressing T synthesis. F5 chemical mix was additive.
21 Cimino-Reale [71]	2008	Cells – rodent femur bone marrow	CFUs; gene microarray; rtPCR ERa, Erβ mRNA	ARS, ATR	Drinking water at levels equal to highly contaminated human exposure: Dams ARS before and all GDs. After birth, male and female pups to 4 months ARS, ATR, or mix	CFU: males decreased with ATR, females increased with mix. Microarray: male 20 genes up regulated by mix only; females 64 genes up regulated by mix. *mRNA:* no effects on ERa whereas Erβ, in males, up regulated by ARS, ATR, and mix; in females only mix significant up regulation

Table 3: (continued)

	First author	Year	Model	Endpoints	Chemicals tested	How mixed	Observed results[a]
22	Kling [72]	2009	Cells – zebrafish liver	Cell viability; proteomics	HBCD, TBBPA, and mix	Graded concentration HBCD, TBBPA, and MIX for cell viability at 24 and 72 h. One middle concentration for proteomics at 72 h	Viablity: single chemicals, graded toxicity was the same at 24 and 72 h, but mix more sensitive to lower concentration at 72 h. Proteomics: identifed 6 up regulated genes, e.g., NADPH generation, PROHIBITIN, Crkl, and 11 down regulated genes, e.g., HSP70kDAa9B, that were unique to the mix.
23	Christiansen [73]	2009	Rodent	AGD, nipple retention, ventral	Dams gavaged DG 7-21, PND 1-16 DEHP, VZ, FIN,	Dose range finding individually, mixes decrease AGD, increase cleft phallus. ×1, ×5, ×0 NOEL	PND 16: additive nipple retention, decrease AGD, increase cleft phallus. Synergistic any malformation. PND47: synergy hypospadias
24	Bermudez [74]	2010	Cells - T47D with ER luciferase reporter	ER reporter gene fluorescence	BPA, BPFA, E2	Mixtures BPA or BPAF with E2	BPA or BPFA add only to low dose E2; no effect BPA or BPFA when E2 above 10 pM E2; no BPA + BPAF additivity
25	Rider [75]	2010	Rodent	Male genital deformity	Gavage pregnant dams BBP, DBP, DEHP, DiBP, DIHP, DPP, Lin, prochl, Procym, VZ, and TCDD in the binary mix only	Corn oil versus 10 chemical mix; binary mix TCDD + DDP	10 chemical mix: addition underestimates with different mechanism; but observed increased genital malformations, e.g., hypospadias, undescended testes. Binary mix only: 50 percent epididymal, testes malformations, malformed external genitalia; liver pathology not seen either alone.
26	Kjaerstad [76]	2010	Cells – AR transfected Chinese Hamster Ovary (CHO) and H295R human adrenocortical cancer	Luciferase reporter of AR activation (or suppression after R1881 agonist); E2 or T in media from H295R cells	Mixtures: 1. AR antagonists – FLT, procymidone, VZ; 2. Inhibitors AR synthesis - Fin, MEHP, prochloraz, VZ; 3. weak antiandrogen - MP, EP, PP, Bp, IBP; 4. Fungicides – epoxiconazole, propiconazole, tebuconazole	Each mixture as its components and as the specific mix	Mixtures 1,2 and 4 supressed R1881 stimulated CHO AR reporter luciferase additively, while mix 4 exceeded additive suppression. Azoles (Mix 4) additively suppressed T synthesis in H295R, but suppressed E2 much less than predicted by addition
27	Hotchkiss [77]	2010	Rodent	Visible morphology PND 2 (AGD), 13 (retained nipples); Necropsy PND 150	PRO (fungicide, AR antagonist), DBP (disrupts T synthesis)	PRO, DBP, or mix each at ED50 GD 14-18; fixed ratio mix and dilutions	PND2: Shorter AGD. PND13: more retained nipples, highest with mix. Necropsy: any genital malformation – mix 55%, DBP 29%, PRO 7%
28	Evans [78]	2012	Cells – MCF7, T47D with ER reporter gene	Proliferation by count of stained cells; reporter gene fluorescence	EE2, E2, coumes, gen, BPA, Narin, BP, BaP, PP, MBC, BP3, tonal, Enterol, Galax, BDE100, MP, Fluoroan, entero	18 chemicals, balanced estrogen effects and nonbalanced mixture attempting measured human ratios	MCF7: additive effects with proliferation assay. T47D: additive effects ER reporter assay

Table 3: (continued)

	First author	Year	Model	Endpoints	Chemicals tested	How mixed	Observed results[a]
29	Boada [79]	2012	Case BRCA:Control	Measure serum organochlorine pesticides			More Ald, Lin, end, DDE, DDD, DDT in cases; DDD remains after adjust for covariables
30	Kim, Y.R. [80]	2012	Cells – GH3 rat pituitary	ERE reporter gene fluorescence; CaBP-9k and PR mRNA and protein	OP, IBP, antiestrogen (ICI182780)	Factoral control and three levels of each without and with ICI182780	ERE, CaBP-9k and PR increase with OP and IBP dose individually, but most mixture effects only with higher dose both OP and IBP. Effects attenuated by ICI182780
31	Kim, S.M. [81]	2012	Cells – GH3 rat pituitary	ERE reporter gene fluorescence; CaBP-9k and PR mRNA and protein	BPA, IBP, antiestrogen ICI182780	Factoral control and three levels of each without and with ICI182780	ERE, CaBP-9k and PR increase with BPA and IBP dose individually, but most mixture effects only with higher dose both OP and IBP. Effects attenuated by ICI182780
32	Brophy [82]	2012	Cases BRCA: control	Occupational exposure			Automobile Plastics OR 2.68 to develop BRCA, Canning food OR 2.35, Bar/Gaming OR 2.28, Agriculture OR 1.36
33	Mlynarcikova [83]	2013	Cells – MCF7	ER-alpha, ESR1; rtPCR Cyclin D1, Cyclin A2, BAX, BCL	E2, BPA, or mix E2+BPA	Alone and binary mixture	Neither affected ER-alpha; low BPA increase ESR1, Cyclin D1, Cyclin A2, Bax and Bcl2 protein; low BPA + E2 additive BAX and BCL2 mRNA
34	Charles [84]	2013	Cells – MCF7	Cell number by Coulter counter	MP, EP, PP, n-BP, iso-BP	Individually and as quinary mixture. All at concentrations measured in human mastectomy specimens	Individual parabens typically had minimal effects, whereas reconstituted quinary mixtures had synergistic effects. Note: all mastectomy specimens had measurable parabens.
35	Scholze [85]	2014	Cells – MCF7	Proliferation	E2, estrone, gen, BPA, DDT, BP, endosul-a, HCH, 3-BC, DDD, ENDOSUL-b, MTC, PP, MBC, DDT, Diel, tonal, OMC, DDD, Ald, HHCB,	Individually and one mix of all 21 chemicals at EC10	Mixture more effect than individual chemicals
36	Orton [86]	2014	Cells – MDAkb2 transfected with androgen responsive luciferase	Androgen receptor blockade as decreased response to standardized dose DHT	One mix of MBC, BaP, BPA, MP, BP, PP, PCB-138, EP, PFOS, VZ, Lin, plus 19 others (list below[b])	EC01, EC10, EC20 doses	No individual effects when each was at EC01; the mixture was active additively
37	Wang [87]	2014	Rat	Mammary terminal end bud Ki-67, apoptosis (TUNEL confirmed by morphology); immunoblotting of protein markers[e]	BPA, Gen, Mix of the two	Pups nursed by lactating dams fed test chemical(s) in drinking water from PND2 – 20; Testing PND21 and PND50	For mix (changes from indiviual chemicals not listed) PND21: more proliferation and less apoptosis, higher ratio Ki-67/TUNEL; lower pAKT, P21, c-caspase-3. PND50: less proliferation and more apoptosis, lower ratio Ki-67/TUNEL; more c-Caspase-9, less p-Akt.

Table 3: (continued)

	First author	Year	Model	Endpoints	Chemicals tested	How mixed	Observed results[a]
38	Delfosse [88]	2015	Cells – HeLa transfected with PxR and luciferase; primary human hepatocytes	Luciferase activation of PxR in whole cells, CYP3A4. X-ray crystallography of receptor binding pocket	40 chemicals but focus on EE2, TNC, E2, and chlor	HeLa: individual and mixed EE2+TNC; EE2+chlor; TNC + E2. Primary hepatocytes: EE2, TNC individual and mix	HeLa cells: Individually EE2 and TNC loose in binding pocket; occupied binding pocket together as combination, i.e., "supramolecular ligand"; PxR synergy EE2+TNC, EE2+chlor, and TNC + E2. Hepatocytes: EE2, TNC minimal effects individually; mix EE2+TNC activated CYP3A4 like the total CYP3A4 activator, SR12813
39	Hadrup [89]	2015	Rodent	Pathology; plasma hormone levels; quantitative – PCR	Gavage - PFNA and mix of 14 chemicals Berga, Glabri, BPA, BP, DBP, DEHP, 4-MB, OMC, DDE, Epoxicon, Lin, Prochlor, Procym, VZz	Fixed mix and different levels of PFNA	Hepatocyte hypertrophy and decreased body weight with higher PFNA. Serum: PFNA and testosterone and dihydrotestosterone increased with low dose PFNA + mix, not PFNA alone. Testis: 17-beta HSD decreased by low PFNA + mix
40	Czarnota [90]	2015	Cases NHL: Control	Chemicals in carpet dust of usual home	Vacuum carpets in homes in four cities; test for 5 PCBs, 7 PAHs, 15 pesticides	Test weighted quantile sum	Varied by city; overall, PCB 180, alpha-chlordane, gamma-chlordane associated with NHL; chlorpyrifos and dicamba inversely related
41	Conley [91]	2016	Cells – T47D with ER luciferase reporter. Rodent – ovariectomize d	Luciferase luminescensce; uterine weight	BPS, MTX, BPAF, BPC, EE2 added to culture cells; gavage to rodents	Mixes BPAF + MTX, BPS + MTX, and BPAF + BPC + BPS + E E2+ MET	T47D: mixes of BPAF + MTX, BPS + MTX, and BPAF + BPC + BPS + EE2+MET all additive. Rodents: mixes BPAF + MTX, BPS + MTX, and BPAF + BPC + BPS + EE2+MET all additive (*in vitro* both under and over estimated *in vivo* effects)
42	Pastor-Barriuso [92]	2016	Case BRCA: control	OR for breast cancer versus estrogenic activity by growth of MCF7 cells	HPLC for specific chemicals; TEXB organohalogenated XE's vs. endogenous hormones and more polar XE's		No single chemical had significant association; TEXB endogenous hormones and more polar XE's associated with increased OR for BRCA
43	Rivero [93]	2016	Cells - HMEC	Proliferation; rtPCR for 68 human kinase and 28 non-kinase genes	Ald, Diel, end, Lin, DDE, DDD, DDT	Two mixes at 1x, 10x, 50x, 100x concentration of test chemicals in the different proportions identifed in BRCA patients *versus* healthy women by Boada (2012). 10x used for rtPCR	Cell survival: increased with both BRCA and healthy mixes at 1x and 10×, but decreased by toxicity of 50× and 100×. rtPCR: BRCA mix caused 40 unique up regulation and one unique down regulation relative to control; BRCA mix compared to healthy women, up regulated GRAF1, BHLHBB, EPHA4, and EPHB2,and down regulated KIT

Table 3: (continued)

	First author	Year	Model	Endpoints	Chemicals tested	How mixed	Observed results[a]
44	Fiandanese [94]	2016	Rodent	Testicle histology, *in vitro* ability to fertilize ova, rtPCR mRNA pituitary and testes	DEHP, PCB 101, PCB 118	mix of PCB 101+PCB 118, DEHP alone, or mixture all three	Intratesticular testosterone lowered by PCBs and DEHP with intermediate result with mixture; FSH and LH mRNA raised by PCBs antagonized by DEHP; FSH-r and Lh-r lowered by DEHP and antagonized by PCBs
45	Routti [95]	2016	Cells – polar bear adipose stem cells; 3T3-L1. Polar bear pbPPARG with luciferase reporter	Phase one adipogenesis: triglyceride levels 3T3-L1 cells. Phase two adipogenesis: transactivation of pbPPARG (luciferase reporter) and triglyceride accumulation	Polar bear liver and fat extracts; 44 brominated and chlorinated POPs identified in HPLC extracts of polar bear fat; sequential HPLC to Mass Spectrometry to identify nontarget chemicals in liver and fat	Concentrated mixtures of 44 POPs, 10 high concentration POPs, 16 methyl- SO2 POP metabolites; and the whole tissue extract	The 44 extracted POPs antagonized pbPPARG as did the 10 found in higher concentration; none of the synthetic mixtures affected adipogenesis whereas adipogenesis was increased by the crude extracts. HPLC-Mass Spec identifed 9 phthalates and 2 other POPs in the crude extract, but not in the synthetic mixture.
46	Guerra [96]	2016	13 day mouse ovarian follicles. Cells - short term human granulosa	Follicle survival in culture and E2 in follicle media; P in granulosa cell media	DEHP, BP, and Mix	Increasing concentration DEHP, BP for individual exposures. For mix: only lower dose BP with two doses DEHP.	Follicles: only mix gave lower E2 in media day 8. DEHP, BP, mix no effect on follicle survival. *Granulosa cells*: DEHP and BP decreased P at 72 and 96 h (only DEHP decrease was significant). Mix restored P synthesis.
47	Seeger [97]	2016	Yeast estrogen screen, CALUX cell luciferase assay	*Yeast*: turbidity. *Cells*:luciferase activation ER alpha and ER beta	Fludioxonil, fenhexamid, CLP	Binary (3) and ternary (1) mixtures	YES: in iso-effective concentrations, 7 of 12 comparisons sub additive, 5 of 12 synergistic. CALUX: additive in iso-effective concentrations
48	Zajda [98]	2017	Cells – benign nonleutinized granulosa cells (HGrC1); Cells malignant granulosa tumor cells (COV434)	mRNA and protein AhR, ARNT, AHRR, COMT, CYP1A1; cell proliferation by AlamarBlue fluorescence; apoptosis by Caspase-3	16 polycyclic aromatic hydrocarbons (PAHs) listed in footnote[c]	Two mixes: M1 – all 16 PAHs; M2 – 5 PAHs thought not to to be human carcinogens (naphthalene, phenanthrene, anthracene, fluoranthrene, pyrene)	Different response benign and malignant cells. Benign cells: at baseline, lower ARNT, CYP1A1, COMT mRNA (lower AhR, higher CYP1A1, COMT proteins); both M1 and M2 increased AhR, reduced AHRR, and increased ARNT. CYP1A1 decreased then net increase. COMT increase more with M2. Proliferation increased with both mixes. Caspase increased transiently only with M1. Malignant cells: M1 and M2 increased ARNT, little effect AhR, AHRR. Both mixes decreased CYP1A1 then return to baseline, COMT increased. Proliferation did not increase. No effect apoptosis.

Table 3: (continued)

	First author	Year	Model	Endpoints	Chemicals tested	How mixed	Observed results[a]
49	Adibi [99]	2017	Cells – human undifferentiated (TBPC) and differentiated (vCTB) trophoblast	CGA, CGB, PPARG mRNA; intracellular hCGB, PPARG; secreted hCG isoforms	MnBP, MBzP, MEHP, MEP	One single level for each phthalate used individually and in mix (used level in urine of pregnant women)	*TBPC*: mRNA effects followed individual phthalates with gender differences in CGB. *vCTB*: intracellular PPARG increased in female and decreased in male trophoblast cells; hCGβ decreased in mixture reversing some phthalates. No other mix effects.
50	Wieczerzak [100]	2018	Cells – HT29 (colon CA)	Cell metabolism (viability) with MTT test; DNA damage with Comet assay	Toxicity of 10 pharmaceuticals found in surface water and/ or lake water (list below[d])	Binary mixtures of four drugs that were cytotoxic by MTT, i.e., diclofenac, oxytetracycline, fluoxatene, chloramphenicol, at 30 percent of the toxic dose	Three doses of binary mixes of each of four drugs gives 54 combinations. Ten were antagonistic, all others additive
51	Ledda [101]	2018	Traffic police case crossover	Spot urine 1-OHP as marker for PAHs; Salmonella mutagenic assay	Urine sample before and after 6 days working as a traffic policeman		After six days, 1-OHP levels doubled and mutagenic activity increased significantly
52	Lee [102]	2018	Cases colon cancer: Controls and subjects with polyps	Serum OCPs and PCBs	*11 OCPs* (HCH, DDT, DDE, Chlor, oxy-Chlor, nanachlor, heptachlor epoxide, heptachlor) and *14 PCBs* (18, 28, 33, 52, 101, 1.5, 118, 138, 153, 170, 180, 187, 194, 199)		Summary measures of the mixture of total persistent organic pollutants and OCPs associated with polyps and colon cancers. Higher PCBs associated with colon polyps but not colon cancer
53	Brinkman [103]	2018	Cells – MCF7 transfected with ER reporter gene	Luminescence from ER activation	Nonylphenols - p353, p363, p262, -p33	Ternary and quaternary mixtures	All are additive, but effects overestimated by common addition methods. P353 added to low dose E2 but antagonized high dose E2. Partial agonists p363, p262 reduced low and high dose E2 effects
54	Dairkee [104]	2018	Cells – three HRBEC cell lines and primary, nonimmortalized HRBECs	S-phase by BUDR incorporation; ERα, ERβ, pERαs118; evasion of TAM induced apoptosis	BPA, MP, PFOA at levels reported in human studies	Ternary mix at human tissue or fluid concentrations, and concentrations one log10 below and one log10 above	Compared to individual chemicals, mixture induced increased S-phase, increased ERα and pERα^{s118}, lower Erβ, and increased evasion of TAM induced apoptosis. Results were confirmed in primary, nonimmortalized HRBECs.
55	Axelstad [105]	2018	Rodent	Testicle sperm count	Human range: ACT, DBP, DEHP, VZ, prochl, POR, LIN, epoxiconazole, DDE, MBC, OMC, BPA, BP,	Dams gavaged GD 7-21, PND 1-22. Used historical controls: no individual chemical data. Mixes 4 estrogenic, 8 antiandrogenic EDCs, and 12 EDCs with ACT	PND 22: increased testicle weight in high dose mixes. PND 300: decreased sperm count epididymis high doses all mixes

Table 3: (continued)

First author	Year	Model	Endpoints	Chemicals tested	How mixed	Observed results[a]
56 Yuan [106]	2018	Cells – MCF7	Cell number by bio-reduction of tetrazolium salt to orange dye; rtPCR	E1, E1, EE2, E3, DES, EV, 4-t-OP, 4-NP, BPA	Two mixtures: 1. all at EC50, and stepwise 2.5 dilutions; 2. a binary mixture of E2+BPA	Responses differ from previous yeast studies by same group. Additive effect for the mixture of nine; in binary mix, mRNA for ERα, IGF, SP- 1, PIK3CA, mTOR and GPER (GRR30) higher in mix; Akt1 lower in mix; Erβ not affected; differ from their previous study with yeast reporter gene
57 Thrupp [107]	2018	Minnows	Egg production	Chemicals with presumed different modes of action: EE2, levonorgestrel, desogestrel, trenbolone, beclomethasone	Five component mixture, all at EC10 level	Individual chemicals caused decreased egg production but mix more so than individual chemicals, e.g.,almost no egg decrease from exposure to low concentrations of individual chemicals, but ~50 percent reduction from mixture
58 Martins [108]	2018	Zebrafish	Comet assay; rtPCR, oxidative stress markers, histopathology	B[a]P and Cd++	Individually and binary mixture: exposed 14 days, then 7 days unexposed	Comet: mix higher day 7, but lower than either singly days 14 and 21, B[a]P comet highest day 14 and 21. mRNA: genes for Detox and DNA repair higher with mix, tp53 higher after end exposure.
59 Conley [109]	2018	Rodent	Fetal testis testosterone production *in vitro*, rtPCR fetal testis custom 96 gene array, examination male pups PND 2, 13, 120	DDE, LIN, Prochl, procymidone, pyrifluquinazon, VZ, FIN, FLT, DPP, DEHP, DBP, DCHP, DEHP, DBP, BBP, DiBP, DiHP, dihexyl and diheptyl phthalates	Gavage dams GD 14-18. One mix LOAEL/5 and dilutions to LOAEL/80. Used LOAELs from literature. Compare to vehicle control.	rtPCR: at 1/5 LOAEL 11 genes up regulated and 9 down regulated. PND 2: decreased AGD. PND 13: retained nipples. PND120:decreased testicle weight and sperm in epididymis
60 Shao [110]	2019	Zebrafish	Mortality of 48 h fertilized fish eggs	BPA, diclofenac, diuron, carbamazepine, penconazole, diazinon, triclosan, cyprodinil, flusilazole, GEN	One mix with each chemical at LC50 and 2-fold dilutions	Effects of mixture higher than individual chemicals
61 Shao [111]	2019	Zebrafish, rainbow trout liver cells (TRLW1); Salmonella typhimurium	Mortality of 48 h fertilized fish eggs; genotoxicity by micronucleus in RTL-W1; Ames histidine reversion assay; *in silico* prediction	Concentrated extracts of samples from 22 Danube River sites; 68 chemicals in high concentration; subset of 29 chemicals with greatest effects predicted *in silico*	Raw river water extracts and a mixture based on *in silico* prediction for 29 components from mixtures at 3 "hotspot" sites	Three "hot spots" water sources were identifed by zebrafish embryo mortality, RTL-W1 micronucleus formation and AMES test on raw extracts. The 29 chemicals selected by *in silico* prediction, accounted for only 48% of RTL-W1 micronucleus of the raw extracts at "hotspots"

[a]Table does not attempt to distingish between concentration addition, response addition, effect summation, synergy, etc. See text.

[b]Additional chemicals for Orton, et al.: fludioxonil, fenhexamid, *ortho*-phenylphenol, tebuconazole, dimethomorph, imazalil, methiocarb, pirimiphos-methyl, cyprodil, pyrimethanil, chlorpropham, benzophenone 2, benzophenone 3, butylated hydroxyanisole, butylated hydroxytoluol, galaxolide, tonalide, BDE100, 3-BC.

[c]PAHs for Zajda, et al.: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoroanthene, pyrene, benz(a)anthracene, CHRY, benzol(a)fluoranthene, benzol(k) fluoranthene, BaP, indol(1,2,3-cd)pyrene, dibenzo(ah)anthracene, benzo(ghi)perylene.

[d]Pharmaceuticals for Wieczerzak et al: diclofenac, ketoprofen, androsteindione, progesterone, etrone, chloramphenicol, oxytetracycline, diazepam, fluoxetine, gemfibrozil.

[e]Protein markers for Wang, et al: ER-α, ER-β, Akt-1, Akt-2, Akt-3, PTEN, p-Akt, Src-1, SRC

Note: For abbreviations see Appendix Table 5.

- Most mixtures behave differently from their individual component chemicals. Mixture effects may be greater than any component chemical but not fully additive, be additive, exceed the sum of the parts (synergy), or occasionally be antagonistic.
- Most mixture research has measured endocrinology endpoints such as ER (or other receptor) levels, proliferation that is ER driven (confirmed by blocking with an anti-ER drug), and tumor formation and genital malformation in animals. Relatively few studies evaluated other endpoints such as tissue interaction, gene mRNA levels, markers of gene damage, cell migration, evasion of apoptosis or AR stimulation, or protein levels. There have been a few case-control human epidemiology studies focused on total endocrine activity related to endpoints such as breast cancer.
- Most studies evaluate mixtures of chemicals with similar modes of action, similar structures, or similar uses such as combined insecticides. This isn't surprising since in 1996 the FDA promoted study of chemical mixtures with similar modes of action [112].
- Rather than human-relevant levels, most studies selected doses to facilitate observing how components of mixtures interact at different dose levels, typically defined in proportion to maximum or minimum effects, with an interest in whether the mixtures exhibited concentration addition, response addition, etc. Few studies added or subtracted components to or from a complex mixture.
- Unconfirmed results limit data usefulness. Over 100 chemicals have been tested, but with exception of BPA, ethinylestradiol, some parabens, DDT (and its metabolites), some insecticides and some phthalates, most results are unconfirmed either in the same or complementary models.

The way forward

Over the past several decades we have made great strides in understanding the biology of cancer. The Halifax Project was the first attempt to use the Hallmarks of Cancer framework as a model to help us consider how different combinations or mixtures of chemical effects might produce cancers. In that effort, the Hallmarks of Cancer [4] were employed as a broad overarching framework to create teams that reviewed key molecular mechanisms and signaling pathways that are disrupted in cancer. Those teams then also reviewed what we know about low dose environmental exposures to "non-carcinogens" to better understand whether or not aggregated mixture effects from seemingly benign exposures might be capable of carcinogenesis.

The idea of an accumulation of individual actions producing carcinogenesis is supported by more recent work by the International Agency for Research on Cancer (IARC) on the Key Characteristics of Carcinogens that categorized the observed effects of individual, single chemicals that are known to be capable of producing carcinogenesis [113]. Some of these Key Characteristics are described using terms that explain general actions of the chemical carcinogens themselves (e.g., act as an electrophile, be genotoxic, etc.) and some of these Key Characteristics are described in reference to the effect these chemicals have on cellular biology (e.g., alter DNA repair, induce oxidative stress, induce chronic inflammation etc.). These categorizations were drawn from studies of chemicals classified by IARC as Group 1, carcinogenic to humans [114]. A subsequent effort to align Key Characeristics with Hallmarks of Cancer [115] noted that the two are conceptually distinct such that Hallmarks describe what biology *exists* in a cancer whereas Key Characteristics describe the actions of carcinogens that *can cause* those Hallmarks to be acquired. There is not a one-to-one relationship of specific Hallmarks to specific Characteristics. The sequence is that carcinogens are "thought to act by inducing multiple Hallmarks in normal cells" and all carcinogens induce one or more Hallmark(s) of Cancer [115].

The theme that emerges from both the Hallmarks of Cancer and Key Characteristic of Carcinogens is that an accumulation of disruptive actions on relevant cellular mechanisms, pathways, and systems can produce cancers. Conceptually speaking, this accumulation of actions could arise from mixture effects produced by individual chemicals that are not carcinogens, from individual chemicals that are carcinogens, or some combination thereof. A related question is whether the actions must accumulate in a specific sequence. Demetriou et al. found the sequence of acquisition of genetic changes in six cancers tended to begin with changes that affected cell number (growth, evasion of apoptosis, etc.) but the exact sequence can vary among those changes [116]. This is slightly different from the concept of latency proposed by Rothman in which the order of accumulating pieces of the causal pie is not specified [48]. Ultimately, however, the challenge of understanding how and in what order these disruptive actions produce the steps involved in carcinogenesis is a problem that remains unresolved. To move the understanding of mixtures forward we must address knowledge gaps concerning the behavior of mixtures:

Assess how all aspects of cell biology respond to mixtures

Most research on mixtures of chemicals has studied how added effects converge on one or a set of endpoints. This concept must be reversed to look from a different perspective at the breadth of cell systems or pathways a mixture might affect. Research must screen *all* metabolic signaling pathways in a cell or tissue in order to determine *whether mixtures trigger unique responses not seen in response to any individual chemical.*

Mixture research has already identified unique effects not seen from the individual chemicals (Table 4) [61, 62, 65, 71, 72, 75, 93] These include unique up or down regulation of genes, unique expression of proteins, neoplastic growth in organs not affected by mixture components alone, and –in proof-of-concept studies – unique gene expression with different combinations of increasing riverine contamination [117, 118].

Expand chemical mixture research beyond reproduction and endocrinology

A century of work with hormonal processes – reinforced by the clinical relevance of hormones and their receptors [119–121] – has provided the framework for most mixture studies to date. This perspective, however, is incomplete because although targeting other Hallmarks is clinically useful, e.g., reversal of immune suppression (pembrolizumab) [122] and blocking second messengers (lapatinib) [123], neovascularity (bevacizumab) [124], cell metabolism (everolimus) [125], or cell proliferation (palbociclib) [126] – little work has addressed how mixtures or even individual chemicals might directly cause or promote these same Hallmarks. Many of these Hallmarks are downstream effects of hormone metabolic signaling pathways, but knowledge that non-physiologic XEs can directly activate estrogen pathways suggests the parallel possibility that other chemicals might similarly activate Hallmarks at points downstream from usual physiologic hormone receptor activation sites.

Empirical information concerning the effects on multiple Hallmarks would have immediate practical application in design of computer models to predict effects of drug and chemical mixtures. Computer models learn from large datasets of empirical observations of representative interactions [110, 127, 128]. When mixture research focuses primarily on hormone related processes, computer models built on that limited background will tend to identify hormone related processes, with less ability to anticipate non-hormone possibilities.

Information on broad-spectrum effects will also help address two additional important questions: First, how do chemicals such as DES and DBP cause cancer years after they have been cleared from blood and urine and exposure has ended? For example, DES is cleared from the body [129] but it causes cancer years after exposure (Table 1). Studies of genetic changes after DES exposure are contradictory [130–133], so the mechanism of how effects persist *after exposure* is an open question. Second, why do chemicals cause cancer only in a minority of exposed persons? In an *in vitro* example using a DES congener, BPA can induce proliferation that persists after it's been removed, but that's a rare event [134] and the mechanism is unclear. This latter observation segues into the broader question of why some people get cancer from exposures, and some do not, i.e., why cancer is a rare event even after exposure to known carcinogens such as DDT, DES, and possibly DBP (Table 1). Understanding a broader spectrum of effects will also help clarify whether and, if so, why Hallmarks must accrue in a specific sequence [116]. Finally, exposure to a legacy mixture of current chemicals will persist indefinitely, and knowledge of mixture effects on all key aspects of normal cell biology will facilitate remediation.

Interpret effects of chemicals in context

The primary concern is how mixtures might affect normal people, so effects must be interpreted in the context of *normal* physiology. During the menstrual cycle, for example, multiple spikes in leutinizing hormone (LH) from the pituitary prompt multiple releases or spikes of E2 from the ovaries [135]. In early childhood, these paired LH then E2 spikes are rare, but they become more common as a child grows and the more frequent hormone spikes induce thelarche and menarche [136]. Additional hormone spikes in young children would disrupt and/or move these processes to a younger age. For example, the xenoestrogen (XE) BPA *spikes* after ingestion and does not accumulate [137]. However, DES – a BPA congener and a carcinogen – does not accumulate either [129]. In theory, XE spikes of estrogenic activity from BPA consumption by children would be of concern because they add virtual, premature, abnormal hormone *spikes* [137]. Research has related early thelarche to BPA exposure in toddlers [138] and girls 4–8 years of age [139], although this has not been observed in older girls at puberty [140] or in all studies [141].

Model tissues of interest

Cancer cell lines, e.g., MCF7 and T47D, are reliable models for endpoints such as additive effects on the ER (Table 3), but established cell lines can be misidentified or carry artifacts introduced over multiple passages [142, 143]. Their greater limitation for studying carcinogenesis, however, is that *malignant cells are already malignant*, they may not react the same as benign cells even if the ER acts the same, and it is conceptually challenging to claim a study has evaluated the transition from benign to malignant cells beginning from malignant cells. It is already known that benign and malignant cells can react differently [98]. Studying cells from non-malignant tissues [53, 61, 71, 72, 84, 91, 93, 96, 104, 111] reduces uncertainty about clinical relevance of results.

Study human-relevant concentrations

Most mixtures research selected doses to facilitate study of how effects of components of the mixture add together, and for convenience, starting doses are often too high to be environmentally relevant. For example, sometimes the ED50 (effective dose that produces 50 percent of the maximal effect) is used as a reference point and compared to higher or lower concentrations, even though all of the studied exposures are above human relevant ranges. Alternatively, researchers may create a mix of chemicals –with concentrations similarly selected relative to maximum effects rather than human relevant exposures –and study dilutions of one mix of chemicals, combined in a fixed ratio, to avoid the permutations of evaluating multiple chemicals in multiple combinations of doses. This method clarifies how effects combine, e.g., synergistic, antagonistic, response addition, etc., but it does not illuminate what happens when the ratios of the chemicals vary [93, 117] or a chemical is added or removed.

An alternative is to remove or add a chemical(s) in a mix using concentrations that have been measured in humans. For example, Charles and Darbre measured five parabens in mastectomy specimens and found each paraben in its individual, human-measured concentration elicited little response from MCF7 cells. However, the same chemicals combined as a reconstituted, human-measured mixture elicited greater than additive cell proliferation [80]. Similar human-relevant mixtures have been based on measurements within the study or values published by others [57, 71, 91, 102, 104, 105].

Plan for future epidemiology

For DES and DDT, groups and specimens, respectively, organized at one point in time – without knowledge of their eventual use – provided the basis for research decades later. Similarly, we should anticipate that our children will encounter challenges we have not imagined. Contemporary collection of biological specimens will enhance future research such as the recent collection of blood and urine samples over three trimesters of pregnancy that has already been a resource for study of mother and child outcomes [144].

Reward research that is not groundbreaking

The ease of interpreting endocrine-based endpoints is rooted in a century of research. As we investigate new Hallmarks and mixtures, priorities must shift to encourage redundancy of studies across models and between laboratories. Confirmation in different laboratories will establish the credibility that ER related effects have earned over a century and promote the *clarity* that arises from an inclusive consensus based on evidence from many kinds of endpoints.

Seek truth through an iterative process

A philosophical barrier threatens progress when science is asked to choose between either 1. testing defined doses and models without a way to prove that any single specific model or group of models provides the definitive answer for all humans, or 2. synthesizing conclusions based on a range of sources of information and experiments. Single large studies may not be as definitive as hoped, and evidence synthesized from multiple perspectives may offer compelling counterarguments [145, 146]. Differences of opinion about how to assess results must be acknowledged between all parties, those who want one kind of data and those who want another. Hopefully, they can arrive at an agreement to proceed without requiring a decision to focus on either single experiments at the expense of overview, or overview at the expense of single experiments.

Go forward now

The research to test the Low-Dose Carcinogenesis Hypothesis from the Halifax Project will be expensive, but the cost

Table 4: Studies finding unique results from mixtures not found after exposure to individual components of the mixtures.

	First author	Year	Model	End points	Chemicals tested	How mixed	Observed results[a]
Studies with laboratory mixtures							
1	Bae [61]	2002	Cells – human keratinocytes	CDNA	As++, Cd++, Cr++, Pb++, MNNG (maximum effect control)	As++ and mixture As++, Cd++, Cd++, PB+ +	Only compared As++ to mix so don't know individual cDNA for Cd++, Cr++, or Pb+ + : 7 genes induced only in mix, whereas 46 genes suppressed only in mix
2	You [62]	2004	Rodent	Gene arrays on PND 90 mammary gland	Gen, MXC	Dams fed Gen, MXC, or Mix from breeding through weaning followed by same for pups the through PND90	83 genes uniquely metabolism, and 42 genes uniquely down regulated, e.g., interleukins and casein kinases, with mix. "…not equivalent to addition of effects associated with GEN or MXC alone."
3	Cimino-Reale [71]	2008	Cells – rodent femur bone marrow	CFUs; gene microarray; rtPCR ERci, Erβ mRNA	ARS, ATR	Drinking water at levels equal to highly contaminated Human exposure: Dams ARS before and all GDs. After birth, male and female pups to 4 months ARS, ATR, or mix	CFU: males decreased with ATR, females increase mix. Microarray: male 20 genes up regulated by mix only; females 64 genes up regulated by mix. mRNA: no effects on ERci whereas Erβ, in males, up regulated by ARS, ATR, and mix; in females only mix significant up regulation
4	Kling [72]	2009	Cells – zebrafish liver	Cell viability; proteomics	HBCD, TBBPA, and mix	Graded concentration HBCD, TBBPA, and MIX for cell viabilty at 24 and 72 h. One middle concentration for proteomics at 72 h	Viablity: Single chemicals, graded toxicity was the same at 24 and 72 h, but mix more sensitive to lower concentration at 72 h. Proteomics: identified 6 up regulated genes, e.g., NADPH generation, PROHIBIN, Crkl, and 11 down regulated genes, e.g., HSP70kDA9B, that were unique to the mix
5	Rider [75]	2010	Rodent	Male genital deformity	Gavage pregnant dams BBP, DBP, DEHP, DiBP, DIHP, DPP, Lin, prochl, Procym, VZ, and TCDD in the binary mix only	Com oil versus 10 chemical mix; binary mix TCDD + DDP	10 chemical mix: addition underestimates with different mechanism; but observed increased genital malformations e.g., undescended testes. Binary mix only: 50 percent epididymal, testes malformations, malformed external genitalia; liver pathology not seen from either alone.

Table 4: (continued)

	First author	Year	Model	End points	Chemicals tested	How mixed	Observed results[a]
6	Rivero [93]	2016	Cells – HMEC	Proliferation; rtPCR for 68 human kinase and 28 non- kinase genes	Ald, Diel, end, Lin, DDE, DDD, DDT	Two mixes at 1x, 10x, 50x, 100x concentration of test chemicals identifed in BRCA patients or healthy women by Boada (2012). 10x used for rtPCR	Cell survival: increased with both BRCA and healthy mixes at 1× and 10×, but decreased by 50× and l00×. rtPCR: BRCA mix caused 40 unique up regulation and one unique down regulation relative to control; BRCA mix compared to healthy women, up regulated GRAF1, BHLHBB, EPHA4, and EPHB2, and down regulated KIT
Proof of principle in aquatic models							
7	Menzel [117]	2009	*Caenorhabditis elegans*; river sediment from the Danube, Rhine, and Elbe Rivers; cells – rainbow trout gonad	Sediment analysis with Gas chromatography , mass spectometry; *C. elegans* with comet assay, YES assay, RNA microarrays	Eight metal ions, DDT, DDD, DDE, HCH, HCB, OCS, PCBs (7)/TBH/TBT	Sediment reconstitued in water with *C. elegans* in concentration according to three river sites	Pollutants:DDT, HCH, HCB, OCS, PCB Elbe > Rhine > Danube. Toxicity: Rhine > Elbe > Danube. YES and Comet: Elbe > Rhine > Danube. Gene expression: 53 up regulated and 56 down regulated uniquely in mix in both Elbe and Rhine in relation to Danube as control
8	Christiansen [118]	2014	Large suckers gathered upper, middle and lower chromatography Columbia River and concurrent river water samples	HPLC followed by gas- chromatography: mass- spectromtry of liver; mRNA microarrays; rtPCR	Correlate mRNA with liver three sites with DDT metabolites(2), OC s(6), PCBs (12), PBDEs (4), CLP, OXFF, TRI	Liver from fish collected at expression: 72 probes progressing contamination	Contaminants: higher in lower river, but overlapped. Gene correlated with one contaminant; 23 probes correlated only with two or more PCBs or PBDEs. rtPCR: confirmed correlation of CES2, CYB5R3, TMED1

Note: For abbreviations see Appendix Table 5.

of ignoring these issues now may be much higher in the future. Specifically, if the cost of preventing cancer is avoidance of a chemical or a mixture of chemicals now, or the cost of preventing cancer is some kind of remediation after exposure but before the cancer develops, we believe taking those steps preemptively will be less expensive than the combined cost of treating the cancer and/or the cost of lost opportunity, life, and income for the persons who develop cancer as has been demonstrated for tobacco control [147]. Such prevention is of value to the general population as demonstrated in a cross-national survey [148].

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Appendix

Table 5: Abbreviations for Tables 3 and 4.

ABBREVIATION	MEANING
1-OHP	1-hydroxypyrene
2,3,4,5 TCB	Tetrachlorobiphenylol
2,4,6 TCB	Trichlorbiphenylol
4-t-OP	4-tert-octylphenol
AAF	Acetylaminofluorine
ACT	Acetaminophen
AGD	Anogenital distance
AhR	Aryl hydrocarbon receptor
AHRR	AhR receptor repressor
ALACH	Alachlor
Aid	Aldrin
ARNT	AhR nuclear translocator
ARS	Sodium arsenate
ATR	Atrazine
BaP	Benzo[a]pyrene
BBN	N-butyl(N-hydroxybutyl)nitrosamine
BBP	Butylbenzylphthalate
BDE 100	Brominated diphenyl ether 100
Berga	Bergamottin
BENZ	Benzanthracene
BHP	Benzylhydroxyparaben
BP	Butylparaben
BP3	Benzophenone 3
BPA	Bisphenol-A
BPAF	Bisphenol-AF
BPC	Bisphenol-C
BPS	Bisphenol-S
BRCA	Breast cancer
CaBK-D9k	Calbindin-D9k
CBP	Chlorobiphenylol
CFU	Colony forming units
Chlor	Chlordane
CHRY	Chrysene
CLP	Chlorpyriphos
COMT	Catechol-*o*-methyltransferase
Cou	Coumestrol
C×43	Connexin 43
DAB	Dimethylaminobenzene
DAI	Daidzein
DBN	Dibutylnitrosamine
DBP	Dibutylphthalate
DCBP	Dichlorobiphenylol
DCHP	Dicyclohexyl phthalate
DDD	Dichlorodiphenyldichlorethane
DDE	Dicholorodiphenyldichloroethelene
DDT	Dichlorodiphenyltrichloroethane
DEHP	Diethylhexylphthalate
DEN	Diethylnitrosamine
DEP	Diethylphthalate
DES	Diethylstilbestrol
DHBP	Dihydroxybenzophenone
DHPN	Dihydroxypropylnitrosamine
DHT	Dihydrotestosterone
DiBP	Diisobutylphthalate
Diel	Dieldrin
DiHP	Diisoheptylphthalate
DMD	Mix of DEN, MNU, DHPN
DMN	Dimethylnitroasmine
DPP	Dipentylphthalate
El	Estrone
E2	Estradiol
E3	Estriol
EE2	Ethinyl estradiol
EHEN	Ethylhydroxyethylnitrosamine
End	Endrin
endosul-a	Endosulfan-alpha
endosul-b	Endosulfan-beta
Entero	Enterodiol
Enterol	Enterolactone
EP	Ethyl paraben
ER	Estrogen receptor
ERE	Estrogen response element
ESR1	Gene for ER-alpha
EV	Estradio-valerate
FIN	Funasteride
FLT	Flutamide
Fluoroan	Fluoranthene
Galax	Galoxolide
GD	Gestation day
Gen	Genestein

Table 5: (continued)

ABBREVIATION	MEANING
Glabri	Glabridin
HCB	Hexachlorobiphenyl
HCBz	Hexachlorobenzene
HCH	Hexachlorohexane
HHCB	Hexahydrohexamethylcyclopentabenzopyran
HMEC	Human mammary epithelial cells (benign)
HRBEC	High risk breast epithelial cell
HSD	Hydroxysteroid dehydrogenase
IBP	Isobutylparaben
IHC	Immunohistochemistry staining
Lin	Linuron
LOAEL	Lowest observed adverse effect level
MBC	Methylbenzylidene camphor
MBzP	Monobenzylphthalate
MEHP	Monoethylhexylphthalate
MEP	Monoethylphthalate
MnBP	Monobutylphthalate
MNNG	Methylnitronitrosylguanidine
MNU	Methylnitrosourea
MP	Methylparaben
MTT	Thiazolyl blue tetrazolium bromide
MXC	Methoxychlor
Narin	Narigenin
NHL	Non-Hodgkins lymphoma
NP	Nonylphenol
OC	Organochlorine
OCDF	Octachlorodibenzofuran
OCP	Organochloride pesticide
OCS	Octachlorostyrene
OHP	Hydroxypyrene
OHPCB	Hydroxylated polychlorinated biphenyl
OP	Octylphenol
OXFF	Oxyfluorofen
P	Progesterone
PAH	Polycyclic aromatic hydrocarbon
PCB	Polychlorinatedbiphenyl
PCDD	Pentachlorodibenzodioxin
PCDF	Pentachlordibenzofuran
PCNA	Proliferating cell nuclear antigen
PFNA	Perfluorononanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonate
phenobarb	Phenobarbital
PND	Post-natal day
PNU	Propylnitrosourea
PP	Propylparaben
PPARG	(nuclear) peroxisome proliferator activated receptor gamma
PRO	Procymidone
Prochl	Prochloraz
PS	Phenyl salicylate
PxR	Pregnane X receptor
resorc	Resorcinol monobenzoate
SIM	Simvastatin
T	Testosterone
TAM	Tamoxifen

Table 5: (continued)

ABBREVIATION	MEANING
TBPC	Trophoblast progenitor cell
TCDD	Tetrachlorodibenzodioxin
TCDF	Tetrachlorodibenzofuran
TEXB	Total effective xenoestrogen burden
TNC	*trans*-nonachlor
TOX	Toxaphene
Tonal	Tonalide
TBT	Tributylin
TRC///L	Triclosan
vCTB	Villus cytotrophoblasts
Vz	Vinclozolin
XE	Xenoestrogen
YES	Yeast estrogen screen

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