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Furthering precision medicine and cancer prevention through novel insights in molecular and chemical carcinogenesis

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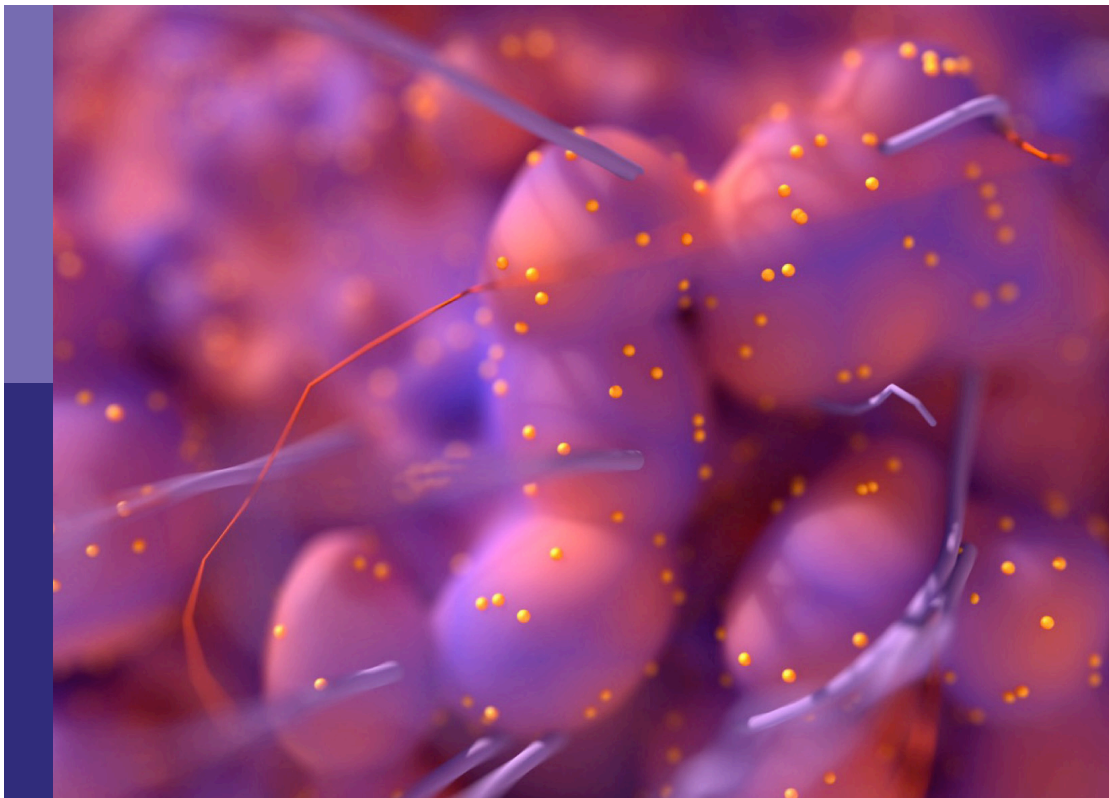

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Furthering precision medicine and cancer prevention through novel insights in molecular and chemical carcinogenesis

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Editorial: Furthering precision medicine and cancer prevention through novel insights in molecular and chemical carcinogenesis

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Editorial on the Research Topic
Furthering precision medicine and cancer prevention through novel insights in molecular and chemical carcinogenesis

A healthy lifestyle, pharmacological strategies, or decreased exposure to environmental carcinogens can reduce risk or delay the development of cancer (1). Several decades of research have explored mechanisms of carcinogenesis, characterized carcinogenicity hazards, and identified novel targets for intervention. Many of these integral discoveries contributed to the identification of the 15 hallmarks of cancer and the 10 key characteristics of carcinogens, KCCs (2–4) Leveraging these findings has the potential to foster the design and the implementation of precision prevention strategies on multiple levels (5).

This Research Topic aimed to produce a collection of articles that discuss known and novel biological targets and biomarkers *in vitro*, *in vivo*, and in human cohorts, including the emerging role of the cancer hallmarks phenotypic plasticity and circulating cell-derived biomarkers. The importance of integrating mechanistic and epidemiological data-driven approaches was highlighted. Particularly, these articles focused on identified early mechanisms of molecular and chemical carcinogenesis aiming to inform precision prevention and to reduce the burden of cancer health disparities.

The complexity of cancer requires a comprehensive approach to understand its diverse manifestations and underlying mechanisms. The Research Topic begins with the perspective article Senga et al. highlighting the need to integrate clear endpoints that anchor KCCs to the acquisition of a complete malignant phenotype into chemical testing. Thus, an all-encompassing strategy that incorporates both evolving KCCs and cancer hallmarks, including the role of the microenvironment, is essential to enable the targeted identification of prevalent carcinogens and facilitate zone-specific prevention strategies. To achieve this goal, collaboration between the KCCs and cancer hallmark communities becomes essential.

Schroeder et al. demonstrated that environmental chemicals with established exposure disparities between non-Hispanic Black women and non-Hispanic White women may influence breast phenotypic plasticity, a new hallmark of cancer. This type of plasticity is associated with basal-like breast cancers typically associated with an aggressive triple-negative subtype which affects African American women at rates of 2-3 times that of White women. These data were largely generated with a high-content imaging microscope demonstrating that innovative techniques will bring us closer to understanding cancer disparities at a molecular level. In addition, to better address and understand breast cancer disparities, it is also important to understand risk among different races. Most epidemiological studies have been performed on non-Hispanic White women. Original research by Patil et al. studied benign breast disease and the subsequent development of breast cancer in African American women.

The identification of extracellular vesicles (EVs) *in vivo* holds potential for the discovery of early biomarkers of carcinogenesis, and/or toxicity endpoints. EVs from donor cells communicate with recipient cells and/or tissues and have the potential to induce toxicity or promote tumorigenesis. However, much of what is currently known about EVs is from studies that perform exogenous administration. A perspective by Nambiar et al. reviews methodologies that track and alter EVs directly *in vivo*, as they are released by donor cells. The authors make the argument that advancements in EV engineering with mouse transgenesis and modern sequencing technologies may provide more insight into this largely unknown area of native EV function and biology. A perspective article by Silver et al. highlights the significance of detecting and characterizing circulating EVs as biomarkers for chemotherapy-induced cardiotoxicity. There are current capabilities to characterize circulating EVs in mice and human liquid cohorts for translational toxicology and cancer medicine.

The perspective article by Hariharan et al. discusses the significance of using human data-driven approaches to improve wellness and reduce tumor recurrence in cancer survivors. Regaining wellness is challenging due to the presence of a myriad of issues induced by radiation, chemotherapy, immunotherapy and/or targeted interventions. This perspective provides promising evidence that global interventions may be possible with data-driven approaches coupled to quality of life and cognitive measurements as well as biological age measurements.

Two original articles examined molecular and carcinogenic effects of a chemical exposure.

Carswell et al. used targeted templated Oligo-sequencing and DNA methylation profiles on a genome-wide scale to identify DNA methylation alterations with early-in-life dichloroacetic acid (DCA) exposure to determine potential mechanisms of liver tumorigenesis. This question arose from previous paradoxical findings whereby DCA exposure shunted cellular metabolism from aerobic glycolysis, which is termed the Warburg Effect and is associated with cancer, to oxidative phosphorylation. Gonzalez-Pons and Bernard examined the interactions between a high-fat diet and benzo(a)pyrene on tumorigenesis in an aggressive mouse model of estrogen receptor negative breast cancer.

Cohen provides a comprehensive review of the different modes of action of chemical carcinogenesis and how to screen for human carcinogens using the primary endpoints of DNA reactivity and mutagenesis, immunomodulation, increased estrogenic activity, and cytotoxicity with consequent regeneration. Mechanistic understanding of chemical carcinogenicity and hazard identification and evaluation will be critical for prevention of future cancers.

In conclusion, cancer prevention strategies will significantly contribute to reaching the goal of the White House Cancer Moonshot Initiative of decreasing 50% cancer mortality by the year 2047 (6). This can be only achieved by bringing together novel ideas, multidisciplinary efforts, and cutting-edge technologies. Hence, this Research Topic provides a collection of articles that will encourage further research and collaborative efforts in the fields of molecular and chemical carcinogenesis for precision prevention and environmental health.

Author contributions

WB: Conceptualization, Writing – original draft, Writing – review & editing. KL: Writing – original draft, Writing – review & editing. JB: Conceptualization, Writing – original draft, Writing – review & editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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A data-driven approach to improve wellness and reduce recurrence in cancer survivors

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For many cancer survivors, toxic side effects of treatment, lingering effects of the aftermath of disease and cancer recurrence adversely affect quality of life (QoL) and reduce healthspan. Data–driven approaches for quantifying and improving wellness in healthy individuals hold great promise for improving the lives of cancer survivors. The data-driven strategy will also guide personalized nutrition and exercise recommendations that may help prevent cancer recurrence and secondary malignancies in survivors.

KEYWORDS

cancer survivor, scientific wellness, long-term effects, late effects, quality of life, dense dynamic personal data, healthspan, cancer recurrence prevention

Introduction: QoL issues in survivors living with cancer and reduced healthspan in cancer–free survivors

Due to advances in early diagnosis and treatment, together with a population with an increasing proportion of older people, the number of cancer survivors in the US is expected to reach 22.1 million by 2030 (1, http://cancerstatisticscenter.cancer.org. Accessed Dec 29, 2023). Most cancer survivors face varying degrees of compromised quality of life (QoL) issues, resulting in reduced healthspan even for those for whom primary treatment was successful and have become cancer-free (2). QoL issues stem from either damage inflicted by the cancer itself, from toxic side effects of therapy (ies) or from a combination of both. While early cancer detection and treatment have improved in recent years, efforts to regain wellness and enhance healthspan in survivors has lagged behind (3). In both survivors living with cancer and in individuals who are cancer-free, optimal wellness goals should include ensuring maximal QoL while preventing cancer recurrence or halting cancer progression. Indeed, it may prove that such goals are well aligned as increasing overall health may help increase resilience to recurrence. As a case in point, in 2022, the American Cancer Society (ACS), after ten years, updated their dietary and exercise recommendations for survivors. At least for survivors of prostate, colorectal and breast cancers, exercise and optimal nutrition appears to

be associated with a lower risk of disease relapse and mortality (4–7). Thus, there's a pressing need to quantitatively transform these observations into personalized lifestyle recommendations for survivors with a view towards especially dealing with cancer complications.

Long-term and late effects are two common types of side effects (8). Long-term effects typically start *during* cancer treatment and *linger on* for weeks to months after termination of therapy (8). In contrast, late effects show up only *after* termination of cancer therapy (2, 9). For example, in some pediatric cancer survivors, later-effects of treatment have manifested several decades after treatment (3) and include cardiac problems.

Here, we briefly discuss some of the more common side-effects of standard cancer therapies before discussing scientific wellness strategies (10, 11) to ameliorating them, reducing risk or early detection of cancer recurrence in survivors.

Long-term and late effects of cancer treatment

Cancer-related cognitive impairment

Of survivors with non-central nervous system cancers treated with chemotherapy, around 40-75% suffer from so-called 'chemobrain' (i.e. 'brain fog') (12, 13). Chemobrain refers to problems with short term memory, inability to focus on a task, impaired information processing, and issues with executive function (12). Such mild to moderate cognitive impairment following chemotherapy, is typically a long-term side effect that lasts for a few months or even years after end of treatment. However, it can sometimes show up as a late effect as well (12).

Several pathophysiological correlates of chemobrain have emerged from recent studies. Some of the more proximal neural correlates with chemobrain come from imaging studies involving cancer survivors. For example, brain imaging studies in cancer survivors have identified a diffuse reduction in white matter and grey matter volume following chemotherapy (14). White matter microstructure also seems to be affected by some chemotherapeutic regimens (14). Other observations in chemotherapy-treated patients include altered activation patterns of cortical networks (15, 16), and changes in brain glucose metabolism that underlie frontal hypometabolism (17). Distal mechanisms have been less well elucidated in chemobrain patients, but studies in mice have shown that commonly used chemotherapeutic regimens can reduce cell proliferation and alter histone modification in hippocampal neural progenitor cells (18). These changes contribute to impaired hippocampal neurogenesis, which can lead to cognitive dysfunction (19, 20). Other studies report that chemotherapy can enhance protein oxidation which in turn activates neurotoxic pro-inflammatory cascades (18, 21, 22). Additionally, genetic polymorphisms in several genes, including those that encode *BDNF* and *APOE4*, have been shown to modulate severity of chemobrain (23, 24).

Chemobrain can significantly disrupt an individual's professional, personal, and social life. There is clearly a need to prospectively identify early and treat cancer survivors who are at high risk of developing chemobrain after therapy. For such high-risk individuals, we need early interventions employed concomitant with initiation of chemotherapy to effectively prevent or at least mitigate chemobrain. Abnormal circulating levels of pro-inflammatory cytokines including Tumor Necrosis Factor-α (TNF-α), its soluble receptor and a few other interleukins have also been correlated to chemobrain in cancer survivors (21). However, reliable clinical biomarkers for chemobrain do not yet exist, and there is not yet a deep understanding of the mechanisms on which to best intervene.

Chemotherapy-induced arthralgia

Post-menopausal women treated with aromatase inhibitors can develop arthralgia — pain and stiffness in their joints, as a long-term side effect of therapy (25). The severity and persistence of arthralgia in these patients is a major factor that drives treatment non-adherence, and in turn leads to shortened survival (26). Exercise regimens and treatment with non-steroidal anti-inflammatory agents (NSAIDS) for managing CIA are typically used in the clinic but with limited effectiveness (25). A possible etiology for CIA is reduced estrogen level triggered by aromatase inhibitor therapy (27). However, a more comprehensive understanding of the mechanisms that underlie CIA are needed to develop effective interventions.

Chemotherapy-induced peripheral neuropathy

Another long-term effect of multiple chemotherapy regimens that contribute to treatment non-adherence is CIPN (26, 28). Onset of CIPN in survivors seems to be dose-dependent; a minority of patients will experience CIPN at moderate chemotherapy doses, but most will only have CIPN at high doses (28).

The pathophysiology of CIPN is believed to be distinct from that of other kinds of peripheral neuropathy (29). Some of the proposed causal mechanisms include chemotherapy-induced mitochondrial dysfunction that triggers apoptosis, altered levels of pain mediators, and aberrant spontaneous activity in A and C fibers (29). While there is no consensus on standard-of-care for CIPN in cancer survivors, a number of medications have shown some benefit in these individuals (28).

Fatigue

Fatigue is a long-term effect and can persist for years after termination of cancer therapy. It reduces QoL and healthspan in survivors and compounds the severity of other side effects (30). Pathophysiological underpinnings of fatigue in cancer survivors include elevated pro-inflammatory activity and insomnia (31, 32).

Obesity and nutrition

Obesity and sub-optimal nutrition in cancer survivors not only influence QoL and healthspan, but also negatively modify prognosis and treatment outcomes (33). Obesity can feed cancer progression and adversely modify some of the other side effects of cancer treatment through multiple mechanisms (33). New pharmaceuticals developed for diabetes treatment such as tirzepatide and semaglutide have recently shown remarkable ability to reduce weight in obese patients without diabetes (34, 35). It remains to be tested how well such interventions might be tolerated in cancer survivors and what the benefit and side effect trade-offs of such treatments might be. Muscle loss and increases in Thyroid cancers are amongst the concerns. Physical activity and nutrition guidelines have been proposed to address obesity and malnutrition in cancer survivors (36). However, since energy balance, metabolism, and nutritional needs for each survivor is different, a personalized, quantitative and continuous approach to address this problem is warranted.

Stress and anxiety

High levels of stress and anxiety are prevalent among cancer survivors, and adversely affect QoL and healthspan (37). The few stress-reduction programs employed in the clinic to date have met with varying degrees of effectiveness (38). As with nutrition, a continuous, personalized approach to monitoring and managing stress in cancer survivors is needed.

Cardiovascular disease and metabolic syndrome

CVD develops as a serious late effect in between 4 to 30% of long-term survivors of childhood and adult malignancies treated with anthracyclines, certain targeted therapies, and radiotherapy (39, 40). Patients treated with anthracyclines are at risk for developing congestive heart failure due to drug-induced cardiomyopathy (41). Cardiac arrhythmias and myocarditis constitute other treatment-related cardiotoxicities (42).

CVD risk in cancer survivors is compounded by metabolic syndrome, which arises from a cluster of factors including dyslipidemia, hypertension, and insulin resistance. Multiple studies have found a higher prevalence of metabolic syndrome in long-term cancer survivors as compared to the general population (43). It has also been suggested that metabolic syndrome in cancer survivors may have a different pathophysiology from that observed in the general population. An altered hormonal axis triggered by radiation- or chemotherapy might partly explain a cancer survivors' increased risk of developing CVD (43).

The presence of specific genetic variants, female sex, and other pre-existing co-morbidities are all risk factors that can predispose cancer patients to developing cancer treatment-induced cardiotoxicities (40). However, biomarkers to prospectively identify cancer patients at high risk of developing treatment-induced cardiotoxicities do not exist (44, 45). The etiology of metabolic syndrome in cancer survivors also needs to be understood in more detail to enable the development of better, more rational interventions.

Secondary malignancies

Treatment-related secondary malignancies are a rare but serious late-effect of certain cancer therapeutic regimens (46, 47). Prospectively identifying survivors at high-risk for developing treatment–associated second cancers can prioritize them for early screening.

Other long-term effects

Lymphedema after surgery (48), and chemotherapy-induced persistent diarrhea are other long-term effects in survivors (49). The role of gut microbiota in modulating irinotecan (an anticancer drug) induced diarrhea has been documented (50). A recent study found that chemotherapy can bring about severe compositional changes in the gut microbiome, in turn triggering intestinal dysbiosis, which eventually leads to gastro-intestinal mucositis (51, 52). The role of the intestinal microbiome in modulating toxic effects of cancer therapy needs to be further explored.

Cancer recurrence

Chances of cancer recurrence depend on various cancer, patient, and treatment related factors including the grade, stage of tumor at diagnosis and, the type of cancer. Recurrence rates range from a low 5% for estrogen positive breast cancers (53) to nearly 100% for glioblastoma multiforme (54), with other tumor types falling somewhere in between. Except for a handful of cancer types, including prostate, chronic myeloid leukemia (CML) and colorectal cancer, useful markers of cancer recurrence do not exist. Dietary and exercise recommendations for cancer prevention often prevent disease recurrence as well. Again, wellness biomarkers that vary longitudinally could serve not only as early biomarkers of recurrence but guides to personalized diet and physical activity recommendations to prevent the complications. Cancer survivors free of disease are excellent candidates to study longitudinally to identify blood biomarkers for early disease reoccurrence. Detecting early re-emergences of cancers when they are simple in their disease complexity, offers an excellent opportunity to explore effective preventive treatments.

Personal, dense, dynamic data clouds as a path to wellness in survivors

Given the myriad issues detailed above for cancer survivors, and their growing numbers, better, and more global, solutions are needed than just attempting to treat individual symptoms. Many

of these can be achieved by leveraging data-driven approaches to enhancing health. The feasibility and utility of gathering longitudinal, multi-tiered data from individuals has been demonstrated in recent studies (10, 55–57) and focused on the development of what we call scientific wellness (58).

In 2014, the first of these cohort studies, the Pioneer 100 Wellness Project (P100) was carried out at the Institute for Systems Biology. The P100 generated personal, dense, dynamic data clouds from a cohort of 108 individuals, sampled on a quarterly basis over 9 months (10). Each participant had their whole genome sequenced as well as longitudinal sampling at 3-month intervals of a large battery of over 100 clinical blood tests, metabolomics and proteomics from the blood, and microbiome from the stool. Additionally, Fitbits were used to track daily activity and tracking of goals and results was monitored and facilitated individually with a health coach. The authors identified thousands of molecular correlates across the various datatypes, including effects of genetics on the metabolome and proteome. Further, when these data were used to guide highly personalized behavioral coaching, participants improved their clinical biomarkers of health and wellness (10).

From 2015-2019, a similar 'scientific wellness' program was offered by Arivale (co-founded by L.H. and N.D.P) that generated longitudinal multi-omic datasets on approximately 5000 people who gave consent for their de-identified data to be used for research purposes. Similar to the P100 study, the program participants, on average, achieved sustained, significant improvements in clinical markers of cardiometabolic risk, inflammation, nutrition, and body mass index (BMI). Improvements in HbA1c levels matched those observed in other landmark clinical trials (59). By finding genetic markers associated with longitudinal changes in such clinical markers, the study also concluded that genetic predisposition impacts clinical responses to lifestyle change in distinct manners for individuals of high and low genetic risk (60).

Subsequent studies using these identified 766 multi-omic associations in the blood related to polygenic risk scores for 54 diseases and traits to provide clues about prevention strategies for prodromal disease (61), discovered early candidate protein biomarkers of cancer and cancer metastasis (62), proposed multi-omic models of biological age as a measure of wellness (55), identified microbiome-derived candidate biomarkers of cardiovascular disease (63) and revealed a key role for the microbiome in healthy aging (Wilmanski et al). Specifically, the Magis et al. study identified carcinoembryonic antigen-related cell adhesion molecule 5 (CAECAM 5), as a persistent, longitudinal outlier, showing up as early as 26 months before individuals were diagnosed with breast, lung or pancreatic cancer. CAECAM 5 is known to be overexpressed in the primary versions of these three cancers and also in metastatic disease. In this case, the scientific wellness data enabled the discovery that such biomarkers of cancer metastasis rose well before diagnosis. Additionally, the study found two additional persistent outliers – calcitonin-related polypeptide alpha (CALCA) and delta-like 1 homolog (DLK1), for metastatic pancreatic cancer. All three proteins would serve as candidate early biomarkers for metastatic disease.

Similar datasets in cancer survivors could provide critical insights to improving health in these populations as well. Actionable discoveries from the scientific wellness approach offer promise to shape choices of intervention for reducing toxic side-effects. This is especially relevant since radiation therapy, hormone therapy and chemotherapy are all known to accelerate biological age in patients (Jacob 64). Their success can be measured using a range of metrics, including objective and subjective measures of QoL, biological age estimates (physiology-based, mutli-omic) and established measures of cognition and wellness. In addition, in cancer survivors, longitudinal phenomic analyses will be useful in detecting reoccurrences early.

Another recent study (56) used integrative omics and data from wearable monitoring to demonstrate the usefulness of longitudinal molecular and physiological deep profiling in precision health. The study authors assayed the genome, transcriptome, proteome, metabolome, immunome and the microbiome, all paired with quarterly enhanced clinical tests, of 109 individuals up to 8 years. These individuals were at elevated risk for developing type II diabetes. Importantly, they were able to discover "molecular pathways associated with metabolic, cardiovascular and oncological pathophysiologies". Additionally, they made 67 clinically actionable discoveries, developed an omics-based model for insulin resistance and fomented healthy dietary and exercise behaviors in the study participants.

The feasibility and stability of building longitudinal, personalized omics profiles of wellness was demonstrated by another recent study in which 100 healthy subjects not only showed the surprising stability of blood plasma protein profiles over time but also their significant association with blood chemistry (57). For example, the authors found a strong association between C- Reactive Protein (synthesized by the liver and a biomarker of inflammation) and Interleukin – 6 (an immune system cytokine related to inflammation). In agreement with other recent studies, the intra-individual baseline variability of omics profiles was low compared to the inter-individual ones.

Thus, longitudinal, high-throughput multi-omic data generation and analysis can be used to (1) define omics baselines of wellness, (2) define early wellness-to-disease and disease-to-wellness transitional omics signatures, (3) discover molecular pathways that underlie specific pathology, (4) find potential biomarkers of disease and (5) help guide and optimize personalized wellness coaching for individuals.

Cancer survivors stand to benefit from such a scientific wellness approach for several reasons as discussed below. Survivors make up a specific group with limited traits, which improves the ability to find meaningful information in a long-term study of large data sets, even with relatively small sample size.

First and foremost, the longitudinal, multi-tiered data collection will enable us to build an integrated baseline in this population. Deviations from this baseline can inform the discovery of novel diagnostic and prognostic biomarkers of various toxic side-effects of therapy discussed above and equally importantly, those of cancer recurrence. For example, data from survivors can be mined to potentially discover new temporal signatures (consisting of metabolites, peptides, proteins or microbiota) that correlate with

known prognostic or diagnostic biomarkers (e.g. PSA and BCR ABL, respectively, for prostate and CML recurrence) (65, 66). The same approach can also be used in survivors to find novel, more effective biomarkers to monitor the effectiveness of maintenance therapy used in some cancer patients even after achieving complete remission.

Second, scientific wellness approaches can be a powerful tool to assess and eventually better tailor, diet and physical activity protocols for survivors with a goal of preventing cancer recurrence and accelerating their return to wellness. This is a corollary to the previously mentioned, new and revised guidelines from the NCI, with regard to diet and physical activity, for cancer survivors.

Third, the longitudinal multimodal data gathering, along with clinical records, will allow us to ask questions around correlative links between other comorbidities like obesity, diabetes or chronic inflammation and cancer recurrence. While the associations between conditions such as obesity and risk of cancer are known, data from our approach will provide a quantitative framework to better assess risks and make new biological discoveries.

Finally, the data clouds from survivors who go on to develop recurrences, could offer insights into tumor evolution from a new point of view (67) and yield new insights into transitions to a state of cancer recurrence. As was true with the other wellness studies, it appears that malignant lesions of the same cancer type, from the same patient are more alike than those from different patients (67).

A longitudinal, big data approach will provide a foundation for future studies in this area, and it will represent a first of its kind resource that can provide a foundation for future studies in this direction. This approach will generate virtual and dynamic clouds myriad data points that provide unique and personalized insights into the wellness and disease of each individual (see Figure 1). We are engaged in two such trials currently focused on cancer survivors: one is a collaboration between Mayo Clinic and Thorne HealthTech and the other is a collaboration between the Institute for Systems Biology and the Swedish Cancer Institute of Providence Health.

As a specific example, metabolomic data for each survivor along with relevant clinical lab data enables monitoring of integral components of metabolic syndrome. Using these dynamic data, a shift towards the metabolic state can be identified and may open up opportunities for early intervention. The data will also allow the effects of physical activity and other medical interventions on the various health metrics of the cancer survivor to be assessed. Other covariates from the data cloud can also be identified in the cohort of survivors, and these will open-up new avenues for intervention and exploration.

Another instance of the translational potential of this approach is the monitoring of pro- and anti-inflammatory cytokines in the blood of cancer survivors. Inflammation is thought to underlie many of the long-term and late-effects after cancer treatment including chemobrain, arthralgia, fatigue, and the metabolic syndrome (18, 25, 32, 43). Therefore, analyzing cytokine levels from each survivor across the cohort will enable discovery of prognostic and predictive biomarkers for these effects.

The longitudinal multi-omic protocol will complement existing, state-of-the-art tumor sequencing methods that provide predictive, prognostic and diagnostic value. Examples of these approaches include the Oncopanel by Eurofin and some other custom panels

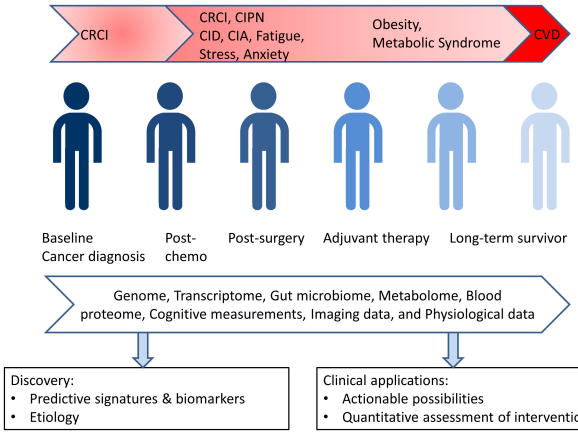

FIGURE 1
A dense, dynamic personalized data cloud path to wellness for cancer survivors.

offered by some cancer centers and clinics in the US (Mayo clinic, MD Anderson and Memorial Sloan Kettering Cancer Center). The scientific wellness approach will bring additional value since it is longitudinal, multi-tiered (not just sequencing) and not tumor tissue specific. The combined value of enriching existing methods that offer a snapshot, with multi-parametric measurements spread over several time points can be evaluated in clinical trials.

Additionally, the dense, dynamic, personal data clouds will contain data from blood proteins which are emerging as potential biomarkers for organ function, especially the heart (45). Importantly, such biomarkers, taken together with physiological data from the same data clouds, will not only yield clues to discover underlying pathophysiological pathways, but will also identify time-points for early medical intervention. Finally, microbiome data from these data clouds can lead to discovery of new associations with disease, wellness, and ways to improve survivor health via the microbiome (68).

We anticipate that moving from carefully selected cohorts to a patient-centric personalized treatment plan will be a multi-step process, requiring carefully planning and evaluation all along the way. The number of studies and patients that would be required to arrive at a clinically translatable recommendation will depend on a variety of factors – robustness of biomarker validation, clinical trial outcomes and other regulatory considerations. The sample sizes provided by the initial exploratory studies provide clues on the effect sizes and can help estimation of the number of participants for future clinical trials.

Concluding remarks

In summary, robust molecular correlates do not exist for most of the long-term and late effects of cancer treatment; the identification of molecular signatures and biomarkers underlying such effects will enable early interventions, and subsequently to their better management in the clinic. Beyond cancer diagnosis and treatment that are rightly the primary focus, further work is needed to improve wellness and quality of life in the increasing number of cancer

survivors, including reduction in their co-morbidities and the early detection of transitions back to a cancerous state. The multi-parameter data from the cancer survivor cohort will allow the generation of sophisticated and highly accurate predictive models for such toxic side-effects and for cancer recurrence. These models can catalyze the discovery of new genetic modifiers for many of the treatment effects, predict high-risk sub-populations, and will help pave a more certain and accelerated path back to wellness for cancer survivors.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

RH: Conceptualization, Writing – original draft. NP: Conceptualization, Supervision, Writing – review & editing. LH: Conceptualization, Writing – review & editing.

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Biomarkers of chemotherapy-induced cardiotoxicity: toward precision prevention using extracellular vesicles

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Detrimental side effects of drugs like doxorubicin, which can cause cardiotoxicity, pose barriers for preventing cancer progression, or treating cancer early through molecular interception. Extracellular vesicles (EVs) are valued for their potential as biomarkers of human health, chemical and molecular carcinogenesis, and therapeutics to treat disease at the cellular level. EVs are released both during normal growth and in response to toxicity and cellular death, playing key roles in cellular communication. Consequently, EVs may hold promise as precision biomarkers and therapeutics to prevent or offset damaging off-target effects of chemotherapeutics. EVs have promise as biomarkers of impending cardiotoxicity induced by chemotherapies and as cardioprotective therapeutic agents. However, EVs can also mediate cardiotoxic cues, depending on the identity and past events of their parent cells. Understanding how EVs mediate signaling is critical toward implementing EVs as therapeutic agents to mitigate cardiotoxic effects of chemotherapies. For example, it remains unclear how mixtures of EV populations from cells exposed to toxins or undergoing different stages of cell death contribute to signaling across cardiac tissues. Here, we present our perspective on the outlook of EVs as future clinical tools to mitigate chemotherapy-induced cardiotoxicity, both as biomarkers of impending cardiotoxicity and as cardioprotective agents. Also, we discuss how heterogeneous mixtures of EVs and transient exposures to toxicants may add complexity to predicting outcomes of exogenously applied EVs. Elucidating how EV cargo and signaling properties change during dynamic cellular events may aid precision prevention of cardiotoxicity in anticancer treatments and development of safer chemotherapeutics.

KEYWORDS

cancer, cardioprotection, cardiotoxicity, chemotherapy, doxorubicin, extracellular vesicles, precision prevention

Abbreviations: DOX, doxorubicin; ESC, embryonic stem cell; EV, extracellular vesicle; iPSC, induced pluripotent stem cell; lncRNA, long non-coding RNA; MI, myocardial infarction; miRNA, microRNA; MSC, mesenchymal stem cell.

Introduction

Rapid, non-invasive strategies for testing and routine monitoring of changes at the cellular level are critical toward understanding chemical and molecular carcinogenesis and preventing symptomatic onset of devastating diseases such as cancer (1, 2). Liquid biopsies, consisting of blood or other bodily fluids, are gaining considerable interest in the scientific community. Biofluids contain a wealth of potential information that could be harnessed toward detecting early cancerous phenotypes (individual or multiple cancers simultaneously) and monitoring response to anticancer therapeutics (3–6). Numerous proteins and nucleic acids, both free-floating and encapsulated within extracellular vesicles (EVs), are released from the cells of internal tissues and are present in extracellular fluids (2, 7, 8).

EVs are a diverse family of membrane-bound particles. EVs were discovered in the early 1980s and first proposed to function as waste transporters for the cell (9). However, coinciding with the finding that EVs contain RNA, the view shifted to consider these particles as potential mediators in cellular communication (10–12). The bi-layered lipid structure of EVs provides a stable means for intercellular transport of a variety of biomolecules, including nucleic acids, both locally and over long distances (13–17). Although a full description of the breadth of cargo identified in EVs would be immense, and is beyond the scope of this article, we point the reader to several excellent reviews and proteomic studies on this topic (18–21).

EVs can participate in cellular communication through fusing with the cellular plasma membrane of target cells and releasing their contents, or through signaling cascades: for example, by binding to receptors on tumor cells to trigger apoptosis (17, 22). EVs display several surface proteins including glycoproteins, tetraspanins, and adhesion molecules that contribute to determining the eventual target and distribution of EVs (17). With their diverse size and composition, EVs play a variety of roles in development and disease. The pleiotropic roles of EVs are well exemplified in the cardiovascular system. Many cells of the cardiovascular system release EVs, including endothelial cells, cardiomyocytes, stem cells, and progenitor cells, both during normal development and in response to disease (23).

EVs may be produced through a variety of mechanisms, including the endocytic pathway (exosomes) (17), budding of the plasma membrane (microvesicles) (24), and as the result of cellular death mechanisms such as apoptosis (apoptotic bodies) (25). These EVs can go on to mediate cardioprotective signaling responses, but EVs can also contribute to cardiotoxicity. Harnessing the cardioprotective properties of EVs and minimizing mechanisms of cardiotoxicity would be of great value for cancer therapies. For instance, many anticancer therapeutics can cause deleterious off-target cardiotoxic effects (26), a classic example being the anthracycline doxorubicin (DOX) (27). In this article, we present our perspective on the potential of EVs as tools to prevent cardiac damage resulting from chemotherapies, both as biomarkers of early cardiotoxicity and as therapeutic cardioprotective agents.

Extracellular vesicles as biomarkers of cardiac health and cardiotoxicity

The deleterious effects of cardiotoxicity caused by some chemotherapeutic agents are multifaceted and include changes in calcium signaling that cause arrhythmias, cardiac hypertrophy, myocardial remodeling, and cellular death (28). Aberrant cellular death in cardiac tissue is of primary concern as it is a driver of cardiac malfunction and disease (29). Cardiotoxicants often lead ultimately to increased cellular death. For example, DOX can trigger numerous cellular death pathways including apoptosis, ferroptosis, autophagy, necrosis, and pyroptosis (30). EVs are of potential value for detecting tissue-level events because they both represent the current state of a given cell and contain cargo representative of past events. For instance, cells that have been exposed to biochemical or mechanical stimuli such as glucose deprivation or stretch release EVs enriched in receptors for glucose or angiotensin, respectively (31). Overall levels of EVs can be an indicator of cardiovascular disease (24). Developing the ability to isolate and trace EV populations back to specific events, such as toxicity, in their parent tissues is an exciting prospect. Circulating EVs could thus serve as early indicators of cardiac malfunction (32, 33), which could be of value for monitoring cardiac health during chemotherapy.

Several EV properties including size, protein composition, and nucleic acids are altered upon cellular demise, providing a potential means by which to communicate tissue-level events such as cardiotoxicity. Cells undergoing a death pathway have the potential to generate very large (>1000 nm) EVs compared to healthy cells. Isolation and characterization of these large vesicles may provide information about the prevalence and pathways of cellular death initiated in response to a cardiotoxic exposure (Figure 1). For instance, apoptotic vesicles can range from 50 -5000 nm (34). Vesicles released from necrotic cells are generally slightly smaller at 200-800 nm (35). EVs of a variety of sizes (30 nm-1000 nm) can be generated in response to several forms of lytic cell death, specifically primary and secondary necrosis and pyroptosis (36). Autophagic processes can generate vesicles (autophagosomes) up to 10 μm in diameter, containing parts of cellular organelles or even intact mitochondria (37).

Further clues as to the origin of an EV may be contained in its associated proteins and cargo. Proteins such as such as CD81, CD63, and CD9 may be indicative of EVs secreted actively via an intracellular pathway (38), whereas vesicles that bud from the plasma membrane during apoptotic or necrotic processes may contain specific cell surface markers (39). For example, both apoptotic and necrotic cells release vesicles presenting phosphatidylserine (35). In addition, EVs expelled from cells undergoing necroptosis, a regulated form of necrosis, carry a key marker of necroptosis, pMLKL (35). Protein cargo can also be significantly altered. For example, a total of 24 EV-associated proteins were found to be differentially regulated in response to apoptosis (40). In addition, EVs generated during apoptosis can encompass fragments of nuclei and thus contain genomic DNA and

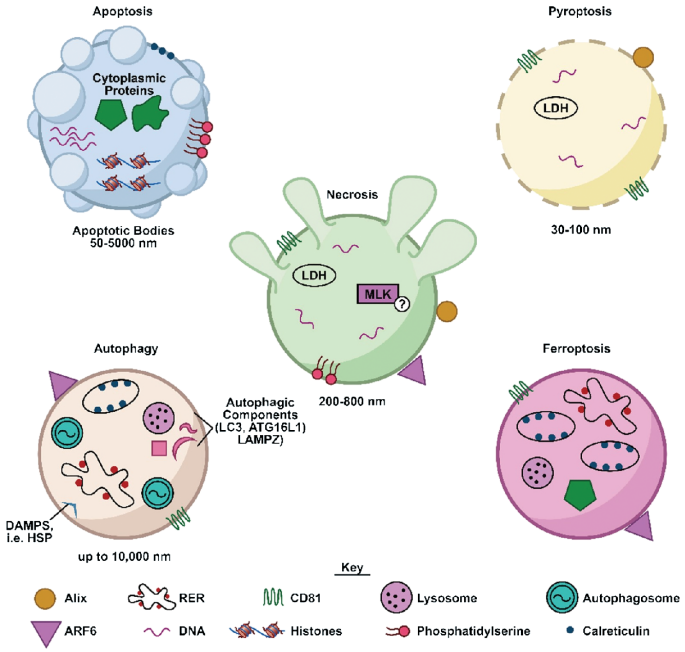

FIGURE 1
Characteristics of EVs produced in response to different cellular death pathways. Shown is a schematic illustrating characteristics of EVs (cargo, surface proteins) released by cells undergoing several different forms of cellular death. DAMPS, damage-associated molecular patterns; HSP, heat shock proteins; LDH, lactate dehydrogenase; MLK, mixed lineage kinase; RER, rough endoplasmic reticulum.

related proteins such as histones, or stem from the plasma membrane and contain cytoplasmic cargo (34). These vesicles were found to differ from EVs released during autophagy, in that their proteome was depleted of nuclear proteins, and enriched in cytoskeletal and mitochondrial proteins (37).

EV-encapsulated nucleic acids provide both a history of events and can confer downstream effects. For instance, the long noncoding RNA (lncRNA) GAS5 was elevated in atherosclerotic plaques in both patients and humans, and EVs containing lncRNA GAS5 promoted endothelial cell apoptosis (41). EVs commonly contain microRNAs (miRNAs), which play numerous signaling roles and may confer gene silencing through mRNA degradation or repression of protein translation (42). miRNAs involved in the propagation of deleterious signaling pathways can be released in EVs from unhealthy or dying cells. For example, oxidative stress caused release of EV-packaged miR-185-5p, which enhances caspase activity and promotes both apoptosis and necrosis (43). Irradiation of human whole blood samples induced upregulation of EV-containing miR-204-5p, miR-92a-3p, and miR-31-5p, which are involved in pathways regulating apoptosis, proliferation, and immune response (44). Although miRNA transfer is believed to be a primary means through which EVs mediate intercellular signaling, they can also signal via cytokines. For instance, cardiomyocytes expel EVs enriched in TNF-α in response to hypoxia *in vitro*, which can go on to promote further cell death in an autocrine manner (30). Although many forms of cellular death protect tissues by eliminating damaged cells, EVs released from dying cells can be a means for propagating deleterious protein machinery within a stressed tissue. For example, autophagy-dependent ferroptosis induced by oxidative stress was found to promote propagation of mutant KRAS to other cell types via EVs (45). Understanding EV-cargo released by cardiac tissues undergoing cellular death in response to toxic insult may uncover EVs that could serve as biomarkers of early cardiotoxicity.

However, programmed cellular death is not necessarily an isolated outcome that reflects toxicity or poor tissue health. Recently, it has been identified that cells can recover from apoptosis after events including caspase-activation, mitochondrial fragmentation, and DNA damage (46). A separate cell death pathway, ferroptosis, was also found to be reversible (47). Mechanisms through which cells can recover from death pathways may serve to protect valuable cell populations such as cardiomyocytes. However, such processes also drive DNA damage, micronuclei formation, and massive genetic rearrangements, which can lead to cancerous mutations and deleterious phenotypes (46). Not surprisingly, RNA transcription was proposed to be a critical step in apoptosis recovery (46). Thus, cells undergoing recovery from a death pathway might be expected to release different sets of EV-encapsulated cargo than homeostatic cells. An important question is whether these vesicles reflect beneficial or deleterious phenotypes. Knowledge of the differences in EVs released from cells that undergo cellular death versus from cells that recover could aid our ability to use EVs as biomarkers. Using released EVs to identify early cardiotoxic and tumorigenic processes could help predict tissue fate and guide preventative therapeutics.

EVs have already shown promise as biomarkers of DOX-induced cardiotoxicity in mouse models. Specifically, DOX treatment was observed to increase serum EVs that were larger

and irregularly shaped, with an increase in protein cargo specific to several key organs including heart and skeletal muscle (48). Notably, these EV-associated protein markers increased earlier and in greater proportions than traditional markers of cardiac disease such as cardiac Troponin-I. In addition to proteins, miRNA within EVs may also reflect DOX-induced toxicity. In dogs undergoing chemotherapy, three serum EV-associated miRNAs were differentially regulated post-DOX treatment (49). Already, such biomarkers have shown potential for clinical utility in humans. Notably, EV-associated miRNA from survivors of acute lymphoblastic leukemia post-DOX chemotherapy were found to be differentially regulated compared to control individuals (50). Further, EV-associated miR-144-3p specifically was correlated with cardiomyopathy. Together, these research efforts suggest that EVs have propensity as biomarkers for cardiotoxicity monitoring. Still, in order for EVs to be successfully implemented as clinical monitoring tools, several challenges must be overcome. Specifically, one limitation is differences in protocols and storage methods for EV isolation across studies, which can alter EV populations and cargo identified (51). Also, the small size and low abundance of some EVs requires increasingly sensitive assays to detect (52). Further development of specific and sensitive methods of EV isolation and detection should bring us closer to identifying EVs that could serve as robust clinical biomarkers. Yet, in addition to using EVs as predictive tools, EVs might themselves be harnessed as cardioprotective agents.

Harnessing extracellular vesicles as cardioprotective agents

Some major goals of EV-based therapeutics are administering cardioprotective EVs immediately post myocardial infarction (MI) (31) to promote cell survival and developing EV-based strategies for inhibiting cardiotoxicity of cancer drugs. Multiple cell types in the heart including fibroblasts, cardiomyocytes, endothelial cells, and cardiac progenitor cells have been found to release EVs that mediate cellular crosstalk, and play numerous roles in cardiac health including protection from atherosclerosis and modulation of inflammation (31, 53, 54). Due to their plasticity, differentiation capacity, flexibility, and renewal capacity, stem cells have been identified as a promising source of EVs with therapeutic potential. Embryonic stem cell (ESC)-derived EVs have been observed to increase survival, proliferation, neovascularization, and reduce fibrosis in mouse MI models (55). 3D cardiospheres, enriched in stem cells, also release EVs that were shown to be protective in MI models (56). Induced pluripotent stem cell (iPSC)-derived EVs have also been shown to protect against apoptosis following oxidative stress and MI (57). EVs derived from mesenchymal stem cells (MSCs) also possess protective properties. Bone marrow MSC-derived EVs may reduce fibrosis and improve cell function and survival (58). Endometrial MSC-derived EVs may be even more cardioprotective than bone marrow or adipocyte MSCs in some contexts (59). Cardiac stem cells pretreated with EVs produced by MSCs performed better in mouse MI models and showed enhanced proliferation, migration, and vascularization in comparison to untreated counterparts through regulation of numerous miRNAs (60). Many miRNAs have been identified to regulate fibrosis through the TGF-β and NF- κB signaling pathways (61). In addition, excellent reviews have been written summarizing the actions of various miRNAs in additional cardiotoxic processes including apoptosis and inflammation (62, 63).

The cardioprotective properties of EVs may be further exploited to deter the cardiotoxic off-target effects of chemotherapeutics such as DOX via several miRNA-mediated signaling pathways. In mice, systemic delivery of cardiac progenitor cell-derived EVs was found to inhibit cardiotoxicity induced by DOX and trastuzumab, a common breast cancer drug, in a manner dependent on upregulation of miR-146a-5p (64). EVs isolated from bone marrow MSCs attenuated the cardiotoxic effects of DOX in rats through delivery of miRNA-96 (30). In addition, ultrasound-targeted microbubble destruction-assisted EV delivery of miRNA-21 to the heart reduced DOX toxicity in mice (65). EVs have been further implicated in the response and mediation of DOX cardiotoxicity through circular RNA spindle and kinetochore-associated protein 3 (circ-SKA3), miRNA-1303, and Toll-Like Receptor 4 (TLR4) (66). Transfer of lncRNAs such as NEAT1 through EVs was also observed to reduce the cardiotoxic effects of DOX in mice via inhibition of miRNA-221-3p (67). Microvesicles released from apoptotic DOX-treated cells also protected mice from later tumor formation (68). In addition, treatment with ESC-derived EVs decreased inflammation and pyroptosis in response to DOX treatment (69). The breadth of cardioprotective capabilities observed in EVs suggests they may have use as therapeutic tools in the clinic. Yet, unfortunately, not only can cardioprotective properties be transmitted by EVs, but also deleterious phenotypes.

Whether an EV will trigger a cardioprotective or cardiotoxic response depends highly on the identity and history of the cells that released them. For example, although EVs from ESCs appear to promote survival and function of cardiac cells following either DOX exposure or induced MI in mice, EVs isolated from embryonic fibroblasts do not show these cardioprotective benefits (69, 70). Rather, EVs collected from fibroblasts can induce cardiomyocyte hypertrophy (71). It is becoming increasingly apparent that EV-mediated signaling is dependent on the differentiation status, microenvironment, and past events of the cells that released them. These factors can profoundly impact the phenotypes transmitted by released EVs, and their ability to promote cardiac tissue health. For example, EVs derived from cardiomyocytes preconditioned by hypoxia or angiotensin have been observed to promote fibrosis by transferring miRNA-208a to fibroblasts (72). In addition to promoting fibrosis or hypertrophy, EVs can serve additional deleterious roles, including cancer drug-resistance via miRNA transfer (73). Strategies aiming to destroy certain cell populations (such as tumorigenic cells) and preserve or repair other tissues (such as cardiac) require an understanding of the numerous and dichotomous roles that EVs can play.

EVs transmit signals which can trigger the survival or demise of cardiac cells, with cell type and microenvironmental cues impacting their release (33). This opens opportunities for generating therapeutic EVs *in vitro* by guiding the phenotype of the cells

from which they are produced. However, the response that will be induced by a given EV type is not necessarily clearly predictable. Intriguingly, for example, prior treatment of cardiac progenitor cells with H_2O_2 to induce oxidative stress enhanced the ability of released EVs to attenuate H_2O_2-induced apoptosis via miR-21 transfer (23). Successful administration of therapeutic EVs also depends on our ability to target these vesicles within the body. For instance, in mouse models, macrophage engulfment of EVs resulted in sequestration in the liver and spleen, preventing them from reaching their intended target tissue (74). Suppressing endocytosis in macrophages by knocking down clathrin, a key protein involved in endocytosis (75), via an EV delivery strategy, improved cardiac targeting of therapeutic EVs containing miR-21a-5p and decreased the cardiotoxic effects of subsequently applied DOX (74). Combining cardiac-targeting peptides with biomaterials can further improve retention of therapeutic EVs in cardiac tissue. For example, encapsulating EVs isolated from human umbilical cord MSCs in a hydrogel containing cardioprotective peptides improved retention and cardiac function post MI in rats (76). In addition, conjugating cardiac stem cell-derived EVs with a homing peptide sequence greatly enhanced their protective ability, targeting them more specifically to the heart (77, 78). Precision targeting of EVs within the body is another critical component of successfully implementing preventative molecular strategies.

Discussion

Current strategies for monitoring chemotherapy patients for cardiotoxicity include echocardiography and molecular biomarkers, most commonly c-troponin and N-terminal-pro-brain natriuretic peptide. However, these assessments may fail to identify the earliest stages of cardiotoxicity, prior to damage taking place (79). Novel biomarkers, such as EVs, are needed toward detection of impending deleterious events. A deeper understanding of EV makeup in response to specific cellular events within a tissue is critical not just for diagnostic purposes, but also for understanding how the balance of EVs impacts surrounding cells and overall tissue health. Since tissues are comprised of multiple cell populations undergoing heterogeneous events, EV tissue-specific signatures are likely complex mixtures of vesicles with diverse cargo and properties. For example, a transient toxic exposure triggering death of cells in one part of a tissue, and subsequent recovery of some cells might be expected to generate a mixture of EVs with cardiotoxic or unknown signaling potential (Figure 2A). The surrounding healthy cardiac tissue would thus be subjected to a mixture of EVs with cardioprotective, cardiotoxic, or unclear cardiac signaling potential. The balance of cardioprotective and cardiotoxic EVs in a tissue would be expected to determine the probable fate of the surrounding cells, but may be increasingly hard to predict in complex EV mixtures (Figure 2B). Further research is needed to better understand EV release in response to transient application of cardiotoxins and recovery from cell death pathways.

Predicting the probable outcome of exposing healthy cardiac tissue to a mixture of EVs is made yet more difficult by the complex relationship between cellular states. For example, cell death is not always a sign of toxicity or poor tissue health. Remarkably, the dependence of tissue repair processes on cell death, termed "regenerative cell death" has been observed in several species and contexts (80, 81). In these studies, EVs from apoptotic cells did not appear to play a toxic role. Rather, the detected apoptotic bodies were engulfed by other neighboring cells (82). However, the extent to which EVs were involved directly in these regenerative processes was not fully explored.

An exciting prospect would be if specific EV signatures could be mapped to individual pathways of toxicity or demise, enabling personalized therapeutic strategies and precision prevention of cardiotoxicity. Toward this end, a better understanding of what types of EVs are released from cells undergoing specific cell death pathways and cells in intermediate stages of cell death would be valuable. In addition, off-target cardiotoxic effects of anticancer drugs can potentially be minimized through encapsulation or parallel administration with cardioprotective EVs. However, to fully develop these strategies, a thorough understanding of the

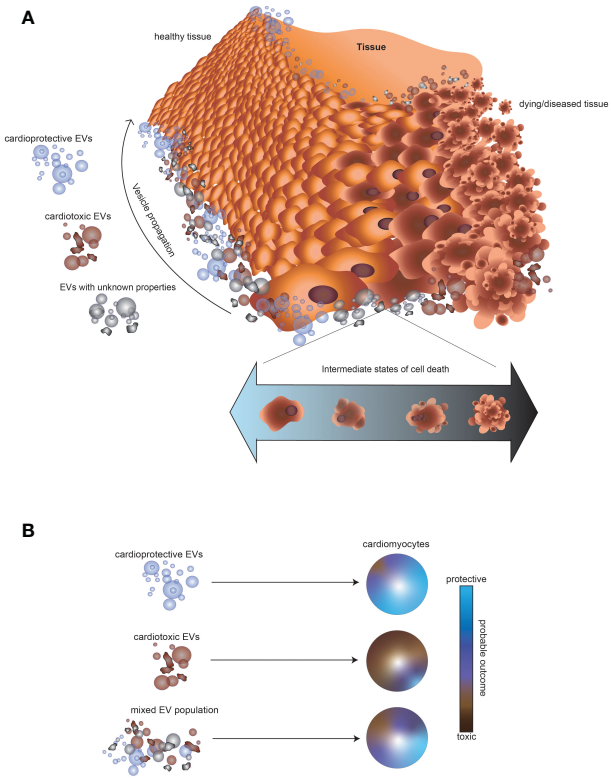

FIGURE 2
Impact of mixtures of EVs released by cells in different states of growth and cellular death on the surrounding tissue. **(A)** Illustration of the possible outcome of a toxic exposure on EV release in cardiac tissue. A transient toxic exposure in one part of the tissue could create a mixture of cell populations: dying cells, unaffected cells, and cells recovering from a death pathway. Consequently, the surrounding tissue would be expected to encounter a mixture of EVs, consisting of vesicles with cardioprotective, cardiotoxic, or unknown signaling potential. **(B)** Schematic illustrating that EVs from some cell types might result in a higher probability of a cardioprotective outcome (blue), whereas other EVs might result in a cardiotoxic outcome (brown). However, the impact of a mixture of EVs with either cardioprotective or cardiotoxic potential is difficult to predict.

factors that contribute to producing EVs with cardioprotective properties is critical. Rather than focusing on simple addition of cardioprotective EVs, a personalized medicine approach balancing the proportions of multiple EV types may increase the probability of a cardioprotective or regenerative outcome.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Author contributions

BS: Conceptualization, Formal analysis, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing. AK: Writing – original draft, Writing – review & editing. MW: Conceptualization, Writing – original draft, Writing – review & editing. KG: Conceptualization, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review & editing. ET: Conceptualization, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

Author AK was employed by Inotiv.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Persistent gene expression and DNA methylation alterations linked to carcinogenic effects of dichloroacetic acid

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Background: Mechanistic understanding of transient exposures that lead to adverse health outcomes will enhance our ability to recognize biological signatures of disease. Here, we measured the transcriptomic and epigenomic alterations due to exposure to the metabolic reprogramming agent, dichloroacetic acid (DCA). Previously, we showed that exposure to DCA increased liver tumor incidence in B6C3F1 mice after continuous or early life exposures significantly over background level.

Methods: Using archived formalin-fixed liver samples, we utilized modern methodologies to measure gene expression and DNA methylation levels to link to previously generated phenotypic measures. Gene expression was measured by targeted RNA sequencing (TempO-seq 1500+ toxicity panel: 2754 total genes) in liver samples collected from 10-, 32-, 57-, and 78-week old mice exposed to deionized water (controls), 3.5 g/L DCA continuously in drinking water ("Direct" group), or DCA for 10-, 32-, or 57-weeks followed by deionized water until sample collection ("Stop" groups). Genome-scaled alterations in DNA methylation were measured by Reduced Representation Bisulfite Sequencing (RRBS) in 78-week liver samples for control, Direct, 10-week Stop DCA exposed mice.

Results: Transcriptomic changes were most robust with concurrent or adjacent timepoints after exposure was withdrawn. We observed a similar pattern with DNA methylation alterations where we noted attenuated differentially methylated regions (DMRs) in the 10-week Stop DCA exposure groups compared to the Direct group at 78-weeks. Gene pathway analysis indicated cellular effects linked to increased oxidative metabolism, a primary mechanism of action for DCA, closer to exposure windows especially early in life. Conversely, many gene signatures and pathways reversed patterns later in life and reflected more pro-tumorigenic patterns for both current and prior DCA exposures. DNA methylation patterns correlated to early gene pathway perturbations, such as

cellular signaling, regulation and metabolism, suggesting persistence in the epigenome and possible regulatory effects.

Conclusion: Liver metabolic reprogramming effects of DCA interacted with normal age mechanisms, increasing tumor burden with both continuous and prior DCA exposure in the male B6C3F1 rodent model.

KEYWORDS

transcriptomics (RNA sequencing), DNA methylation (5mC), dichloro acetic acid, mouse model, age, tumorigenesis, liver

Introduction

The current default approach taken by the US Environmental Protection Agency (US EPA) when considering life-stage exposure is that cumulative exposure averaged over a lifetime is to be considered health protective when assessing exposure risk (1). However, high exposures may occur over a short-term window. In these cases, averaging these exposures over a lifetime may be inappropriate. It is therefore important to understand the linkage of life-stage stressors and latent adverse health outcomes. There are many examples of non-genotoxic exposures that increase susceptibility to cancer later in life (2–6). The mechanistic basis for this persistence can be multi-factorial, however the role of epigenetic dysregulation has increasingly been recognized as a common factor linked to cell proliferation, survival and invasion, immune response, genome instability, and energetics (7, 8) and has been proposed as an important hallmark of cancer. Therefore, a better mechanistic understanding of the epigenetic contribution to cancer biogenesis is a needed.

Dichloroacetic acid (*syn.* dichloroacetate, DCA) provides an interesting case study of latent carcinogenic effects from early-life exposure in mice. DCA is a halogenated acetic acid with low volatility that has previously been assessed by the US EPA because of its stable presence in drinking water as a byproduct of disinfection (9) and potential carcinogenic metabolite of the ground water contaminant, trichloroethylene (10). In male and female mice, as well as male rats, continuous exposure to DCA significantly increases the incidence of liver adenomas and carcinomas (9, 11–14), which led to the US EPA classifying it as a likely carcinogen. However, DCA is also recognized as a metabolic reprogramming agent and has been explored as therapeutic for diabetes, lactic acidosis, lipid/lipoprotein disorders, pulmonary arterial hypertension, and interestingly, for cancer (15). Given that typical amount of exposure in drinking water is much lower than calculated risk levels in humans (13, 16), it is unlikely these doses of concern are achieved due to environmental exposure. Therapeutic doses can reach much higher levels [25 mg/kg-day; (17)] and long-term deleterious effects could be of more legitimate concern, in addition to other recognized adverse effects such as peripheral neuropathy (18).

DCA-mediated mode-of-action for rodent liver cancer outcome is not entirely clear. The structure of DCA resembles that of pyruvate, which can bind and inhibit the activity of pyruvate dehydrogenase kinase [PDK; (19)]. PDK phosphorylates and subsequently inhibits the enzymatic action of the pyruvate dehydrogenase (PDH) complex, which is responsible for the conversion of glucose-derived pyruvate to acetyl-CoA. Activation of PDK reduces pyruvate shuttling to mitochondria (and subsequent acetyl-CoA-mediated activation of the TCA cycle) and instead favors pyruvate conversion to lactate. With DCA exposure, PDK is inhibited thereby activating the PDH complex and shunting glucose to the mitochondria and oxidative metabolism (20). This chemically induced bioenergetic shift can persist after exposure, therefore DCA is considered a metabolic reprogramming agent. However, it was also thought that this forced shift to mitochondrial oxidative metabolism may also be the cause of long-term oxidative burden, leading to cell injury, secondary DNA damage, and eventually cancer (21).

Importantly, our previous research confirmed that not only persistent DCA exposure but also short-term exposure early in life resulted in increased liver tumor burden (13, 22). These persistent tumorigenic effects occurred regardless of length of exposure length tested (ranged 4 wks to 93 wks). Most measured hepatic effects were transient, including mild hepatocellular necrosis, inflammation, and pigmentation, which resolved by 5 weeks after DCA exposure was removed (23). More persistent effects such as hepatocellular hypertrophy and cytoplasmic vacuolization (evidence of increased glycogenic storage) resolved by 26 weeks after DCA cessation. Overall, there was not strong evidence for classical genotoxic or non-genotoxic modes of action for latent carcinogenicity, leading to the hypothesis that persistent epigenetic alterations may be contributing to these latent effects. In this study, we utilize targeted RNA-sequencing and genomic-scaled DNA methylation methods using archived samples from this study to better understand the mechanistic basis for persistent effects of a brief carcinogenic exposure early in life.

Methods

The study utilized archival frozen and formalin-fixed paraffin-embedded (FFPE) samples that were ≥ 30 years old. Due to use in prior studies, we lacked frozen samples for all time points with the exception of 10 week Stop and Direct groups at 78 weeks (Figure 1), which were utilized for Reduced Representation Bisulfite Sequencing (RRBS, *see Methods below*). Gene expression was measured from FFPE samples using a target-based RNA-sequencing method that had been previously utilized to replicate findings in FFPE samples compared to matched frozen samples in a sister study of similar archival age (24). Because of the limitations of these lower quality samples, we used stringent criteria to identify potentially erroneous findings and, as a result, some groups had reduced sample numbers due to these quality control restrictions.

Study design

As previously published, male B6C3F1 mice were treated with 3.5 g/L DCA in deionized water in a stop exposure study design over a period of 78 weeks (23). Mice were either provided only deionized water (control), continuous DCA (direct) or in a series of stop-dosage timepoints after which the mice were switched to deionized water for the duration of the experiment. The Stop group treatments were as follows: 10 weeks of DCA followed by 68 weeks of water (S1) which equates to an exposure period of early childhood thru adolescence, 32 weeks of DCA and 46 weeks of water (S2) which equates to an exposure period from early childhood to a mature adult and 57 weeks of DCA with 21 weeks of water (S3) which equates to an exposure period from early childhood to late middle age. The Direct group were exposed to 3.5 g/L for the entirety of the 78-week study (senior mice). At each timepoint (10, 32, 57 or 78 weeks), a subset of the control, Direct and Stop treatment block mice were humanely euthanized with CO_2 following EPA approved Animal Care and Use Committee protocols. Portions of the livers were fresh frozen at each sacrifice timepoint along with the preparation of FFPE paraffin blocks. Figure 1 outlines study collection points and treatments. FFPE-derived samples were used to measure gene expression and frozen samples were used for DNA methylation determination.

Animal care

The sourcing, housing, and maintenance of the mice in this archived study have been extensively detailed in prior publications, along with dosing details and animal behavior notes (13, 22, 23). All procedures involving animal care were approved by an EPA Institutional Animal Care and Use Committee. Briefly, mice were received at age 21 days to the EPA AAALAC International accredited facility from Charles River Laboratories in Morrisville, NC, and acclimated for 7 days prior to treatment initiation. The animals were housed in polycarbonate cages in a laminar flow room and maintained under standard conditions (20 ± 2 C°; 40–60% relative humidity; 12 h light/dark cycle) and fed *ad libitum* Purina 5001 laboratory rodent diet (Purina Test Diets, St. Louis, MO). The study animals were monitored daily for health impacts. Water intake and body weight were recorded daily.

Drinking water dosage

The target dose of 3.5 g/L (429 mg/kg-day) DCA (CAS 79-43-6, Aldrich, Milwaukee, WI) was based on the earlier carcinogenicity results observed in male mice (13, 22), and is equivalent to 65 mg/kg-day in humans (23). Although this dose was up to approximately 10^5 times greater than DCA concentrations measured in finished drinking water samples (33–160 µg/l) (9) and approximately 2.6 times greater than doses used clinically in studies of children and adolescents with congenital metabolic diseases (12.5 mg/kg every 12 h) (19), this dose captured greater than 85% of the tumorigenic

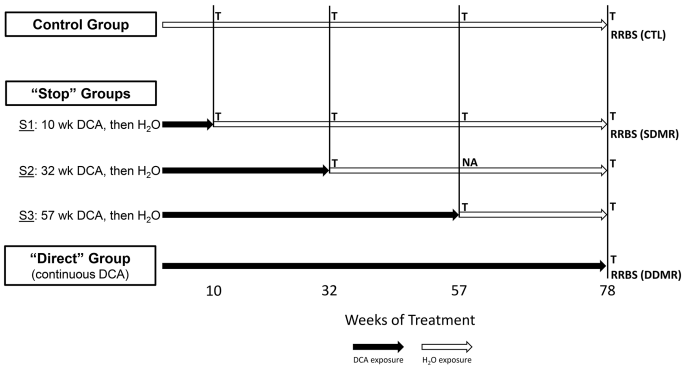

FIGURE 1
Study groups and sample overview. The stop-treatment mouse study design consisted of samples from four time courses of DCA exposure. The control group was given drinking water *ad libitum*, whereas mice in the "Stop" groups "S1", "S2", and "S3" had 3.5g/L DCA added for 10, 32, and 57 weeks, respectively. The "Direct" group was given DCA in drinking water for the length of the study (78 weeks). Liver gene expression was measured at each of these time points with the exception of "S2_57" weeks, where archived samples were not available (NA). The 1500+ TempO-seq assay was used to measure gene expression in FFPE samples indicated as "T". Differentially Methylated Regions (DMRs) were determined by Reduced Representation Bisulfite Sequencing (RRBS) at the 78-week time point for the control (CTL), "S1" Stop group (SDMR) and the Direct group (DDMR) using frozen liver tissue.

effect observed with lifetime exposure at the same 3.5 g/L dose (22). The actual dose was estimated based on intake, body weight and water DCA concentrations, and the daily water concentration (ml/kg-day) by cage was measured every 4-5 weeks beginning with week 4. Drinking water stocks were prepared every 12-14 days and checked by ultraviolet absorption at 231nm to confirm DCA concentration (Beckman DU Series 600 spectrophotometer, Beckman Coulter, Brea, CA). The stock water pH was adjusted to 6.8-7.2 with NaOH, stored at 4-6°C and changed every 5-7 days, based on studies showing DCA to be stable over 1-2 weeks (11, 14). As previously detailed, water consumption was initially 5-22% lower in the DCA drinking water groups, although by 52 weeks, water consumption was equivalent in all groups. The removal of DCA quickly returned water consumption to control levels, regardless of exposure length.

DNA isolation

Approximately 25 mg of frozen liver tissue was cut on dry ice and immediately placed into a 2 ml vial with ceramic homogenization beads (MP Biomedicals, Lysing Matrix D) containing 360 µl of Qiagen buffer ATL (Qiagen GmbH, Hilden, Germany). The tissue was allowed to equilibrate to room temperature, 20 µl of 40 mg/ml Proteinase K was added and homogenized with a Precellys 24 homogenizer (Bertin Technologies, Villeurbanne, France) at 5500 rpm for 20 s. The homogenized sample tubes were placed in a 56°C thermomixer at gentle agitation for 3 h. After proteinase treatment, the samples were treated with RNAse A, and DNA was isolated using Qiagen's DNeasy® kit following the manufacturer's instruction. The isolated DNA was checked for initial purity (A260/280 of ≥ 1.8, A260/230 ≥ 1.0) using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware). Yield was determined by Qubit broad range dsDNA assay kit and protocol (Life Technologies, Carlsbad, CA).

Reduced representation bisulfite sequencing

DNA samples isolated from the frozen liver tissue (n=4-6) were analyzed for methylation on a genome-scale using a modified Reduced Representation Bisulfite Sequencing (RRBS) method (25, 26). Briefly, 500 ng DNA was digested overnight with MspI (300 ng) or TaqαI (200 ng). Each sample underwent an additional 2 hr digestion before restriction enzyme deactivation. Each restriction enzyme digested DNA sample was pooled and then bead purified using Agencourt Ampure XP beads (Beckman Coulter Life Sciences, Brea, CA), and Qubit quantitation was used to determine the amount of lambda (λ) for bisulfite methylation efficiency spike-in. End-repair and adapter ligation were performed with the KAPA Hyper Prep kit (Roche, Basle, Switzerland), along with a custom synthesized duplex adapter (Integrated DNA Technologies (IDT), Morrisville, NC). After overnight ligation and subsequent bead cleanup, the samples went through two rounds of bisulfite conversion and clean-up (Qiagen Epitect Bisulfite conversion kit, Germantown, MD). Library amplification was carried out using KAPA HIFI HotStart Uracil+ and IDT synthesized index and universal primer for 12 cycles, followed by bead cleanup. Library quality and yield were determined with the Agilent Bioanalyzer HS DNA kit (Santa Clara, CA), with the library size range predominately between 300 and 600bp. The libraries were sequenced on an Illumina NextSeq (San Diego, CA) by the National Health and Environmental Effect Research Laboratory (NHEERL) Genomics Research Core (GRC) at the US EPA using the NextSeq 500/500 75 cycle kit. Base calls were based on a Q-score of 30 for greater than 85% and a pass filter of ≥25M reads per sample. RRBS FASTQ files are available from NCBI GEO accession #GSE242665.

Differential methylation analysis was carried out using Bismark alignment (27). Briefly, sequencing adapters and read pairs with a mean Phred quality score of <20 were removed from reads using Trim Galore! (v 0.2.8). Bismark (v 0.14.3) aligned the reads to mm10 mouse genome assembly. Methylated and unmethylated cytosine were extracted from each CpG site and methylation percentages were derived, excluding any loci that were <10 reads and/or contained a known single nucleotide polymorphism. Average methylation percentages were calculated for the control, Direct and S1 78-week group samples by dividing the count of methylated reads by the total number of reads at each covered CpG site. Retained averaged methylation percentages contained >10 reads. All samples exhibited good bisulfite conversion rates (> 99%), alignment (>60% mapped to unique reference), and base quality scores (>96%, average = 98%). Differentially methylated regions (DMRs) were based on clustering of differentially methylated CpGs compared to control samples, which was used to generate DMRs based on the weighted methylation percentages from all CpGs in each region, as previously described (26). The final DMR list contained at least 3 CpGs within each DMR and an independent two-group Student's t-test (p<0.05).

DMRs were mapped to regulatory features and transcription factor binding sites sourced from the Mouse Ensembl Regulatory Build, filtering for "liver:a8w" (release 90). CpG features were sourced from the UCSC annotation track database (ftp://hgdownload.cse.ucsc.edu/goldenPath/mm10/database/). Ensembl, Entrez and MGI identifiers were downloaded from BioMart data mining tool (https://useast.ensembl.org/info/data/biomart/index.html) to allow interconversion of identifiers. All DMRs for the Direct and Stop groups were linked to a single gene ID list based on nearest transcription start site using the R/Bioconductor package, GenomicRanges (28). Riken, GM- and pseudo genes were omitted from any gene or gene pathway subsequent analyses.

FFPE derived mRNA sequencing

Archival samples were processed at BioSpyder, Inc. (Carlsbad, CA) using targeted Templated Oligo-Sequencing (TempO-Seq Mouse S1500+ Surrogate Assay, Mouse Tox v1.1, 3044 probes, 2755 genes) a liver-centric, sentinel gene, probe-based panel designed for high throughput predictions in toxicology (29). TempO-Seq performs well with older RNA-fragmented samples as compared to traditional RNA-Seq (24). Measurements were taken from direct lysates of archived

mouse liver sections derived from FFPE blocks. Two to three sections of 15 μm thickness from each sample block were closely trimmed to remove excess paraffin and placed into -20°C storage until shipment overnight on dry ice to BioSpyder for library generation. Lysate purification, library preparation and sequencing were performed at BioSpyder using an established protocol (30). After passing quality control checks for total mapped reads in positive control samples (>1.5M mapped reads, we observed 4.6M reads), signal-to-noise ratio of the number of mapped reads in positive controls versus negative controls (>20:1, we observed 2077:1), and percentage of mapped reads in positive controls (>70%, we observed 95.9%), sequence data were aligned and matched to the probed gene for the assay. Gene count data were provided to EPA for further analysis. TempO-Seq FASTQ files and count data are available from NCBI GEO accession #GSE242665.Additional bioinformatic filtering was carried out to ensure quality of the data, necessitated by the age of the FFPE blocks (20+ years) and degradation of the resultant RNA. First, excessive deviation of gene expression ranking in individual samples from the average was assessed by Spearman rank regression. Any sample that exhibited an R^2 value of less than 0.7 was excluded from further analysis (10 of 78 samples). A minimum *n* of 3 was required to keep a sample timepoint, although the majority of timepoints ranged from 4 to 6. Second, after median ratio normalization, genes with very low expression were eliminated from further analysis (those <3.4 (20%) geometric mean counts across all samples) as the final filter. Using these filtered samples and normalized genes, we determined differentially expressed genes from age-matched controls using DESeq2 (significance based on FDR ≤0.05) in Partek Flow software environment (Build 10.0.22.0524). A minimum fold change of greater than +/- 1.5 was set as an additional cut-off. Uniform Manifold Approximation and Projection (UMAP) was utilized as a general non-linear dimension reduction to visualize the first 15 components of Principal Components Analysis (PCA) for filtered, normalized count data before DEG determination, thereby capturing 99% of the variation of the filtered S1500+ gene expression data.

Further gene pathway analyses sets were carried out using Ingenuity Pathways Analysis (IPA) (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis). Some visualizations were generated using R packages Plotly for bubble chart generation (v.4.10.1 https://github.com/plotly/plotly.R), VennDetail for Venn and UpSet plot generation (v 4.3 https://github.com/guokai8/VennDetail), and shinyCircos for Circos plot generation (v 3.3.3, https://github.com/venyao/shinyCircos).

Results

Gene expression overview

We determined differentially expressed genes (DEGs) of the treatment groups for all time points compared to the age-matched Controls Group (Figure 2; Supplementary Data 1). In general, the number of DEGs were greater the closer the measurement was to the DCA exposure window and then declined after DCA exposure was stopped regardless of the initial dosage duration. Groups with longer DCA exposures (S3 and Direct) had significantly more DEGs at 78-weeks compared to the shorter exposure durations (S1 and S2). This was similar to what was observed with select gene expression following DCA removal in our earlier study (23). We also observed a "peak" in DEGs for the timepoints measured following DCA removal for the S1 and S3 groups 22 and 21 weeks after exposure cessation, respectively. Archived samples were not available for S2-57 weeks group to confirm a similar peak in DEGs as that observed for the S1 and S3 groups. The majority of the DEGs were specific to the direct DCA exposure period, mouse age, and time after exposure. Only one gene, *Cavin2* (*Sdpr*), was differentially expressed in all timepoints. Some DEGS, such as *Irs1*, *Ctnnd1* and *Ebp* have the same expression trend over all timepoints but failed significance (FDR-corrected *p*-value <0.05) in some treatment groups. Others are altered in only specific treatment subgroups, such as *Col1a1*, *Col4a1* and *Cebpb* in S1 and S2 samples.

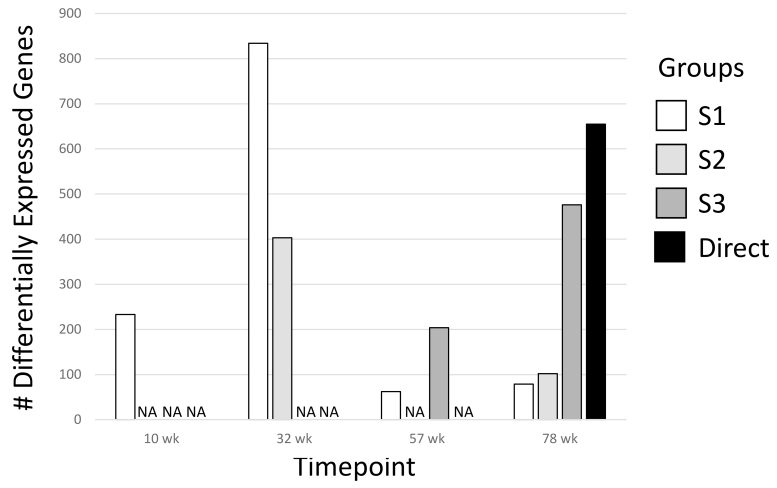

FIGURE 2
Differentially Expressed Genes (DEGs) for all groups. The 1500+ TempO-seq gene expression assay was used to determine differentially expressed genes compared to time-matched controls. DEGs were determined from 3-6 mice using a False Discovery Rate (FDR)-adjusted p-value of ≤0.05. All experimental groups were represented (see Figure 1), with the exception of S2-57 weeks, where samples were missing from the archive (NA, not available).

Ingenuity Pathway Analysis (IPA) was utilized to examine gene pathway enrichment for DEGs of the different treatment groups (Supplementary Data 2). In S1 and S2, early acute phase and inflammatory pathway signaling were upregulated initially, while many pathways linked to oxidative stress and known DCA effects were predicted to be downregulated (*sirtuin signaling, Hif1a signaling, unfolded protein response, oxidative phosphorylation*). At 78 weeks, enriched gene pathways were linked to long term cellular damage (*apelin liver signaling, wound healing signaling, intrinsic prothrombin activation, and GP6 signaling*), despite the relatively short exposure periods as compared to S3 and Direct groups. Enriched pathways differed for the S3 and Direct groups. Almost all significant pathways in the Direct group were predicted to be downregulated (*cholesterol biosynthesis, unfolded protein response, TGF-β signaling*). The S3 group contained multiple pathway alterations by 78 weeks, many of which were linked to predicted reduction in xenobiotic metabolism and enhanced signaling of crucial developmental and regeneration liver factors such as β-catenin and HIF1α. *NRF2 mediated oxidative stress response* was significantly down regulated in both S3 and Direct groups by 78 weeks. Of note, the activity of the lipid homeostasis regulator PPARα was predicted to be downregulated with early DCA exposure (S1-10) and up-regulated with later persistent DCA exposure (Direct group at 78 weeks; Supplementary Figure S1).

DNA methylation alterations overview

Annotation and characterization of differentially methylated regions (DMRs) are summarized in Supplementary Data 3. There were considerably more DMRs in the Direct group (DDMR; 3814 regions) compared to the Stop group (SDMR; 662 regions), likely attributed to diminishing effects of DCA with time away from exposure. This distinction was evident for all chromosomes (Figure 3A; Supplementary Figure 2). DMRs were mapped to predicted regulatory regions and CpG features in the mouse genome (Figure 3B). Although the overall numbers of DMRs were reduced in the Stop group samples, the percentage linked to promoters and CpG islands were roughly twice that of the DDMRs. Strikingly, the DDMRs are predominately hypomethylated (78.5%

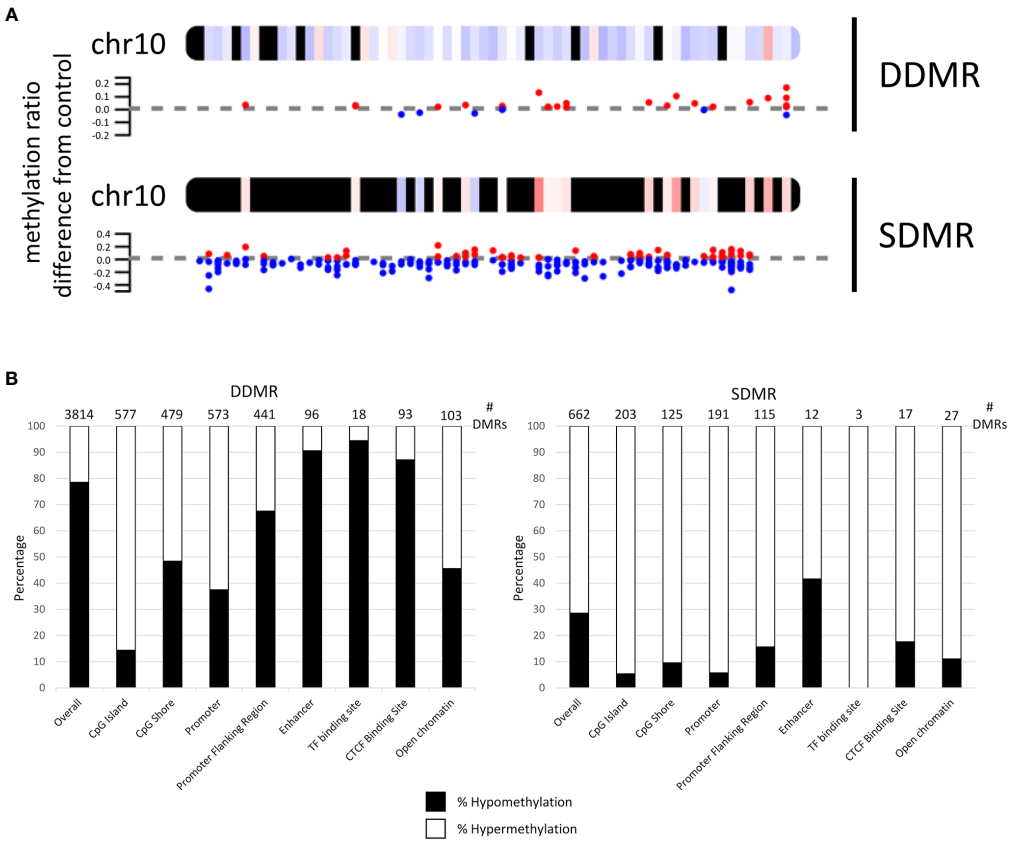

FIGURE 3
Mapping of differentially methylated regions for Direct (DDMR) and Stop (SDMR) 78-week measures. **(A)** Representative mapping DMRs for Chromosome 10. Red and blue dots represent hyper- and hypomethylated regions, respectively, as defined by calculations provided in *Methods*. Averaged intervals of hyper- or hypomethylation are represented by a gradient scale of red-to-blue, respectively, on the chromosome map. *Y-axis* represents the ratio of methylation differentiation compared to control. All chromosomal maps can be seen in Supplementary Figure 2. **(B)** DMR intersection with identified CpG and regulatory features in the mouse genome. Bar graphs represent the percentage of hyper- and hypo-methylated regions in different CpG and regulatory regions that are identified by Ensembl Regulatory Build (release 90; http://useast.ensembl.org/info/genome/funcgen/process/humanandmouse/annotation.html).

TABLE 1 Overview of major gene family differences in the DDMR as compared to controls.

Gene family function	Associated genes*
Extracellular Matrix Function and Regulation	*Adam*, ***Adamts***, *Cdh*, ***Col***, ***Fgf***, *Fndc*, *Sgcb*
Development, Metabolism and Cellular Regulation	***Adcy***, *Ankrd*, ***Ccdc***, *Cdk*, ***Dnah***, ***Fam***, ***Fbxl***, *Fbxo*, ***Hox***, *Klhl*, ***Lrrc***, ***Mrps***, ***Prr***, ***Rpl***, ***Sema***, ***Trim***, ***Usp***, ***Wnt***, ***Zdhhc***
GCPR and Rho GTPase Cell Signaling Activity	*Adgrl*, ***Arhgap***, *Arhgef*, *Dock*, ***Gpr***, ***Rab***, ***Rgs***
Transmembrane Signaling and Transport	***Atp***, *Cacna*, ***Epha***, *Galnt*, *Kcna*, *Kctd*, ***Kif***, ***Pcdh***, ***Rnf***, *Robo*, ***Slc***, *Slitrk*, ***Syt***, ***Tmem***
Gene Regulation	*Ddx*, ***Fox***, ***Parp***, ***Prdm***, *Snord*, *Wdr*, ***Zfp***, ***Zscan***

* Bolded genes are also associated in the SDMR.

overall), while the SDMRs are predominantly hypermethylated (71.5% overall). Genes were linked to DMRs based on nearest transcriptional start site (TSS). With this characterization, 35% percent of the SDMR-linked genes intersected with those linked to DDMRs (Supplementary Data 3). Many members of major gene families that are dysregulated in liver disease (e.g., cancer) were linked to altered DNA methylation patterns in the DDMR group (Table 1) and partially in the SMDR group (bolded genes).

Related, gene pathway analysis using IPA indicated 101 significant pathways were enriched with DDMR-linked genes, while only 15 were enriched with genes linked to SDMR (Supplementary Data 4). Eight of the 15 SDMR-linked gene pathways (53%) intersected with DDMR-linked pathways (Figure 4). These intersecting pathways are involved with regulation of cellular physiology and metabolism, such as G-protein coupled receptor and S100 family signaling pathways. Interestingly, we observed enrichment of the neurotransmitter-linked signaling pathways, GABAergic receptor and glutaminergic receptor signaling pathways.

Time course analysis of continuous and persistent effects of DCA exposure

To explore age and treatment interactions for both continuous and previous DCA exposures, we divided the analysis into three subgroups (Figure 5). First, we grouped all the timepoints collected while the animal was continuously exposed to DCA (S1-10, S2-32, S3-57, and Direct-78), referred to as the "Continuous Exposure" subgroup. The second subgroup analyzed the persistent effects of DCA after 10 weeks of exposure before stoppage (the S1 group progression: S1-10, S1-32, S1-57, and S1-78), referred to as the "Stop" subgroup. The last subgroup, "78-week", consisted of all the 78-week timepoints (S1-78, S2-78, S3-78, and Direct-78) and is most representative of the cumulative DCA mediated changes over time for both continuous and persistent effects. DMR-linked genes as part of these subgroup comparisons and a master table of all DEG and DMR-linked gene intersects, including gene pathway associations, are listed in Supplementary Data 5.

Continuous exposure subgroup analysis

The unique nature of each timepoint for both controls and continuous DCA treatments was demonstrated by UMAP (Figure 6A) with the most divergent groups seen early at 10 weeks, both with and without DCA exposure. Approximately half or more DEGs induced by DCA exposure were unique to a particular timepoint (Figure 6B). Only 2 DEGs were shared across all four timepoints (*C9*, *Sdpr*), which increased to 18 DEGs when comparing timepoints S1-10, S2-32, and 78-week (Direct). DEG intersections between 10-/32- and 78-week (Direct) timepoints were 70 and 80 DEGs, respectively. When comparing shared DEGs between the Direct group and S1-10, S2-32, or S3-57, the percentages were 48%, 43%, or 45%, respectively, demonstrating some degree of commonality between earlier and later DCA exposure related effects. Strikingly, most of the DEGs shared between S1-10 and 78-week (Direct) timepoints demonstrated opposite expression patterns suggesting an important age interaction with continuous DCA

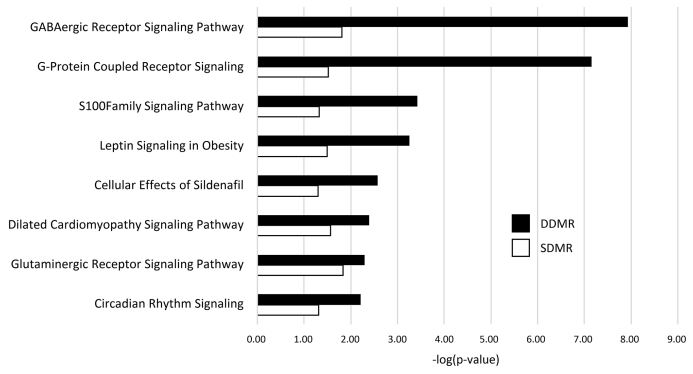

FIGURE 4
DMR-linked genes enrichment of canonical gene pathways. Genes linked to DMRs in both the Direct (DDMR) and Stop (SDMR) groups by mapping to the nearest Transcriptional Start Site (TSS). All gene pathways that were enriched in both groups are graphed. Enrichment was considered significant if Fisher's Exact test -log (*p*-value) was greater than 1.3.

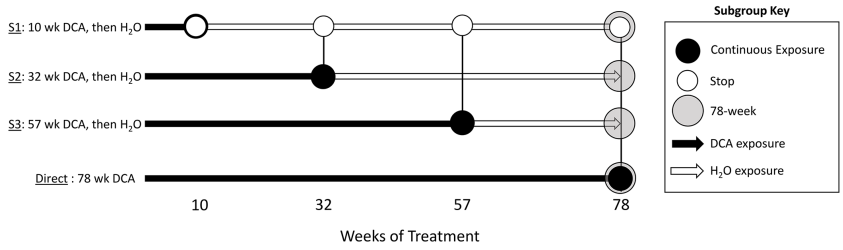

FIGURE 5
Overview of subgrouping of study timepoints. We defined the "Continuous Exposure" subgroup as samples from mice that were continuously exposed to DCA (S1-10, S2-32, S3-57, and Direct-78; black-filled dots. The "Stop" subgroup consisted of samples collected from mice exposed to 10 weeks of DCA only at all collected timepoints (the S1 group progression: S1-10, S1-32, S1-57, and S1-78; white dots). The "78-week" subgroup consisted of all the samples collected at 78 weeks (S1-78, S2-78, S3-78, and Direct-78; grey-filled dots).

exposure. This "flip" in directionality is also demonstrated at a gene pathway level, where S1-10 pathways are primarily activated, and 78-week (Direct) pathways repressed within or across pathway categories (Figure 6C). The intermediate timepoints (S2-32 and S3-57) showed a mixture of predicted activation and repression states. The cellular stress and injury pathways were a clear example of this drift over time, suggesting a switch from acute stress response to liver dysfunction and cancer.

The DNA methylation assessment at 78 weeks may not only reflect the current gene regulatory state, but also previous

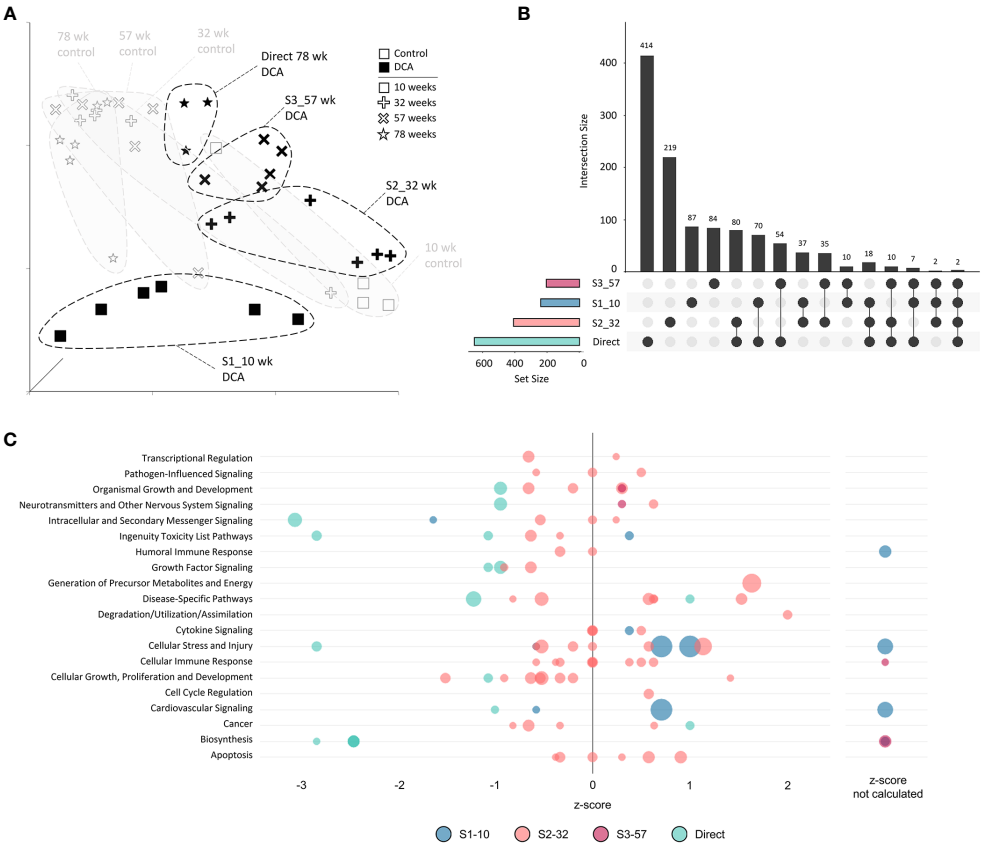

FIGURE 6
Continuous exposure subgroup differentially expressed genes and enriched canonical gene pathways. **(A)** Uniform Manifold Approximation and Projection (UMAP) for the "Continuous Exposure" subgroup samples. UMAP is based on 15 Principal Components of normalized count data before DEG determination, which captured over 99% of the variation displayed in these samples. **(B)** UpSet plot demonstrating differences and similarities of significantly (FDR-corrected p-value<0.05) altered genes compared to time-matched controls. Connected dots identify the group(s) that match to the adjacent bar and DEG number unique for those samples. **(C)** Bubble graph representing gene expression pathways enriched by the "Continuous Exposure" subgroup. Each bubble represents an individual canonical pathway, calculated by Ingenuity Pathway Analysis (IPA), that are categorized by larger meta-categories along the y-axis. The right-to-left placement identifies predicted pathway activation or repression (positive or negative z-score, respectively). Some pathways did not have the data to generate this prediction (no z-score). The size represents the -log(p-value) of the pathway enrichment. The color indicates the experimental mouse group that the pathway enrichment was measured.

perturbations to the epigenome at earlier timepoints. Due to lack of archived samples available for this study, we could only assess DNA methylation at 78 weeks and, instead, used the DEGs measured at all the collection time points as a proxy for gene activity that may be influenced by epigenetic state. Given DEGs from the Continuous Exposure subgroup were derived from mice in which DCA exposure was not removed, the DDMR data were the best comparator. The overall number of DEGs intersecting with DDMR-linked genes (as defined most loosely by the closest gene TSS only or more stringently by further refining DDMRs also located within predicted gene regulatory regions) were low (Table 2). Despite these low intersections, we observed DEGs at all timepoints reflected in the DDMR-linked gene list. When combined across timepoints, 11 DDMR-linked genes (TSS and regulatory region defined) overlapped with DEGs in the Continuous Exposure subgroup that were also part of significant gene pathways at the collected timepoints (Figure 7, Table 2 last column). These genes were included in pathways mediating inflammation, oxidative stress response, and mitotic signaling/regulation, with many downregulated and linked to hypomethylation at 78 weeks. Because we observed this overlap in DDMR-liked genes and DEGs that play roles in pathways known to be perturbed by DCA exposure in liver, this suggested that DDMRs present at 78 weeks reflected gene regulatory patterns observed, at least in part, by earlier and current DCA exposure.

Stop subgroup analysis

We next examined the gene expression persistence and DNA methylation after only 10 weeks of treatment to better understand the carryover effects of early DCA exposure. The UMAP visualization summary of gene expression across the S1 Stop group demonstrated the similarity of the 10- and 32-week timepoints and clear separation to later timepoints and age-matched controls (Figure 8A). The data indicated that S1-57 and S1-78 samples grouped closer to controls, however, separation is still evident indicating persistent gene expression effects well after cessation of exposure. As mentioned earlier, S1-10 and S1-32 DEGs were more numerous than the later S1-57 and S1-78 group DEGs. The largest DEG intersection was between the S1-10 and S1-32 groups (136 genes), where 58% of S1-10 DEGs were shared (Figure 8B). At a pathway level, S1-10 and S1-32 groups also tended to enrich similar categories, in particular cell stress and injury and toxicity pathways (Figure 8C). Pathways associated with S1-57 and S1-78 tended to be repressed, with the notable exception of activated cell stress and injury pathways at 78 weeks. Conversely, related pathways of immune response and cytokine signaling tended to drift from an activated state to a repressed state over time.

The SDMR methylation measures were derived from the S1-78 sample group, and similar to the DDMR analysis for the Direct group comparisons, we believed the SDMR data could reflect both the epigenomic state of the matched timepoint and persistent alterations from earlier time points. To assess this, we examined the intersect of S1 Stop subgroup DEGs and SDMR-linked genes as defined by TSS only or by TSS and proximity to mouse gene regulatory regions at all time points (Table 3). Overall numbers of intersects were low, due to the limited number of total SDMR-linked genes, but those shared genes that mapped to pathways tended to demonstrate downregulation at earlier timepoints and upregulation at the 78-week timepoint (Figure 9). Downregulated genes were primarily hypermethylated in S1-10 and S1-32 treatment groups and have roles in HIF1a signaling, senescence, cancer, and adipogenesis. The upregulated gene *(Hes1)* at S1-78 was hypermethylated and involved in the *Vitamin D Receptor/RXR activation* pathway.

78-week group timepoint analysis

Finally, we assessed if varying DCA exposure durations altered the gene expression response at 78 weeks by comparing all the 78-week sample DEGs. UMAP visualization of the 78-week subgroup DEGs demonstrated separation of treatment groups compared to the 78-week aged control (Figure 10A). S1-78 and S2-78 groups demonstrated higher variability among individual samples, compared to the S3-78 and Direct-78 week, which exhibited the most similarity to each other. This was also reflected in the degree of DEGs overlap between the later timepoints (S3-78 and Direct treatment groups; 201 genes, Figure 10B), possibly reflecting the continuum of changes due to lengthy DCA exposure with age and metabolic status. As noted previously, DEGs are greatly reduced in the S1-78 and S2-78 groups, likely due to *near* return to cellular homeostasis over time after DCA exposure. Of note, 70% and 77% of the S1-78 and S2-78 DEGs, respectively, intersected one or more longer exposed samples, indicating persistent DEGs are similar to responses observed in more continuous exposures. Gene pathways linked to the Direct and S3-78 treatment groups are primarily

TABLE 2 Continuous exposure subgroup and DMR-linked genes intersection.

	DEGs	TSS-only Defined		TSS and Reg Region Defined	
		DEG/DDMR Gene Intersect	DEG/DDMR Intersected Genes in Pathways	DEG/DDMR Gene Intersect	DEG/DDMR Intersected Genes in Pathways
S1-10	233	23	3	16	2
S2-32	403	53	16	25	5
S3-57	204	15	1	11	1
Direct	655	53	8	28	4

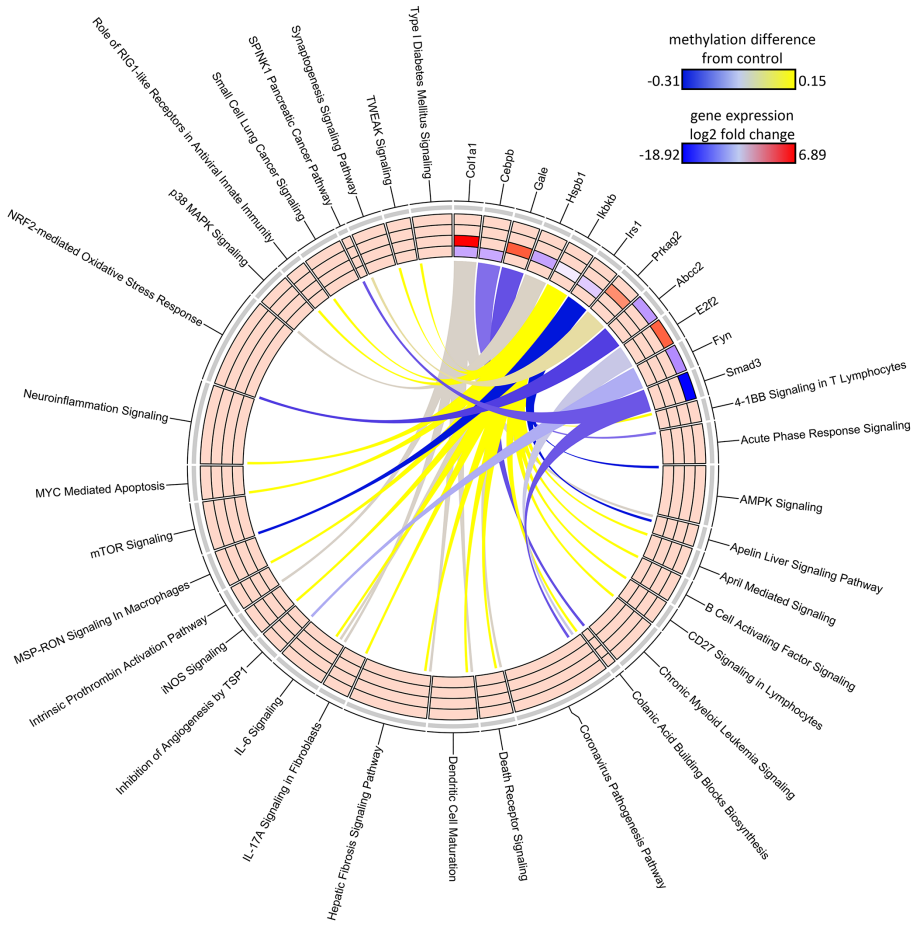

FIGURE 7
Circos plot of DDMR genes linked to "direct" group gene pathways. Connections denote DDMR-linked genes with canonical gene pathways that are enriched by DEGs identified in the Direct groups. Color gradient of connections denote degree of methylation change compared to control mice (yellow = hypermethylated, purple = hypomethylated). Tracks denote $\log_2$ fold-change (red = upregulation, blue = downregulation) of gene expression compared to time-matched controls for S1 10-week, S2 32-week, S3 57-week and Direct 78-week sample groups (inside -> outside). Slice size for individual pathways is proportional to number of DEGs that link to each individual pathway.

repressed, especially for continuous DCA exposure (Direct 78-week group), and linked to toxicity, cell stress and injury, and metabolism (Figure 10C). Pathway enrichment analysis of S3-78 DEGs indicated activation of neurotransmitter signaling and cellular growth, where both S3-78 and Direct groups demonstrated activation of cancer pathways. Pathway enrichment of S1-78 and S2-78 DEGs suggested activation of cellular stress and repression of immune pathway categories.

While genes linked to DDMRs and SDMRs were respectively derived from Direct 78-week and S1-78-week samples, we assessed if there were any commonalities with other 78-week samples. By percentage, the S1-78 and S2-78 group DEGs, which represented earlier-in-life exposures and longest periods of persistent gene alterations, demonstrated the larger overlap to DDMR-linked genes (Table 4). Specifically, 8.9% (7 of 79) of S1-78 and 8.8% (9 of 102) of S2-78 intersected DEGs with DDMR-linked genes (defined by proximity to TSS and also within a gene regulatory region). This is compared to 5.0% (24 of 476) in S3-78 and only 4.3% (28 of 655) in the Direct 78-week group. These overlapped DEGs also demonstrated a larger proportion mapping to significant canonical pathways in the sample groups with longer time since DCA exposure compared to group with more recent exposure (Table 4, *last column, DDMR rows*), providing some evidence that these genes linking to direct DCA-mediated methylation changes are known components of pertinent gene pathways that persisted well after DCA exposure. As previously noted, few S1-78 DEGs intersected with SDMR-linked genes. A greater number of DEGs of the longer DCA exposures overlapped SDMR-linked genes, but this is likely due to chance because of the larger overall DEGs with the samples of more recent DCA exposures.

Discussion

Persistent effects of chemical exposures that reprogram the metabolic profile of cells can have important impacts later in life. In liver tumors, cancer cells favor glycolysis versus oxidative phosphorylation even in the presence of oxygen, a process known as aerobic glycolysis or the Warburg effect (31). This cancer-mediated metabolic programming is thought to maintain an ideal

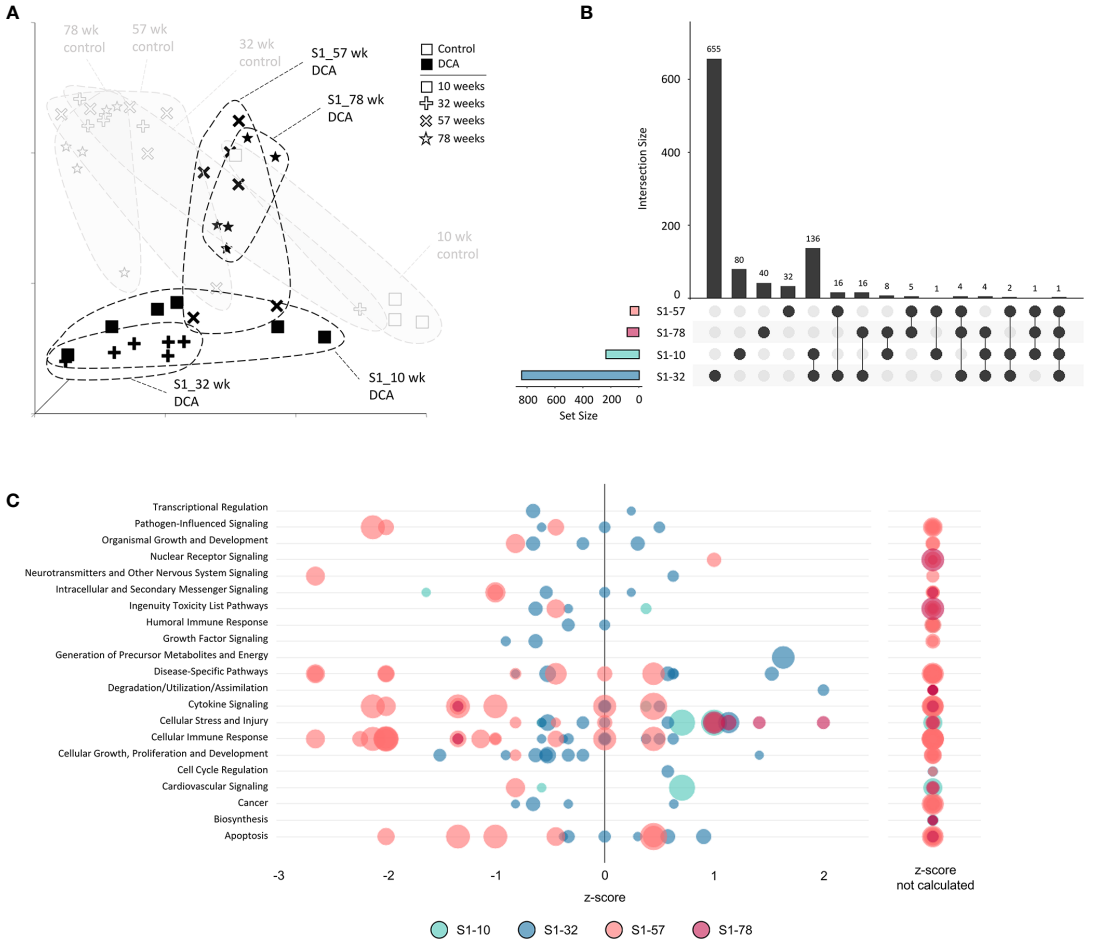

FIGURE 8
Stop subgroup differentially expressed genes and enriched canonical gene pathways. **(A)** Uniform Manifold Approximation and Projection (UMAP) for the "Stop" subgroup samples. UMAP is based on 15 Principal Components of normalized count data before DEG determination, which captured over 99% of the variation displayed in these samples. **(B)** UpSet plot demonstrating differences and similarities of significantly (FDR-corrected *p*-value<0.05) altered genes compared to time-matched controls. Dots identify the group(s) that match to the adjacent bar and DEG number unique for those samples. **(C)** Bubble graph representing gene expression pathways enriched by the "Stop" subgroup. Each bubble represents an individual canonical pathway, calculated by Ingenuity Pathway Analysis (IPA), that are categorized by larger meta-categories along the y-axis. The right-to-left placement identifies predicted pathway activation or repression (positive or negative z-score, respectively). Some pathways did not have the data to generate this prediction (no z-score). The size represents the -log(*p*-value) of the pathway enrichment. The color indicates the experimental mouse group that the pathway enrichment was measured.

environment for tumor initiation and growth by reducing mitochondrial-derived reactive oxygen species (ROS) production and apoptotic pathway activation, maintaining the genomic stability of stem cells, and maintaining a favorable acidic microenvironment through lactate production (32–34). DCA, a pyruvate analogue, shunts metabolism toward oxidative phosphorylation thereby promoting ROS production, anti-growth signaling and apoptosis through mitochondrial cytochrome c

TABLE 3 Stop group DEGs and DMR-linked genes intersection.

	DEGs	TSS-only Defined		TSS and Reg Region Defined	
		DEG/SDMR Gene Intersect	DEG/SDMR Intersected Genes in Pathways	DEG/SDMR Gene Intersect	DEG/SDMR Intersected Genes in Pathways
S1-10	233	8	2	7	2
S1-32	834	17	6	9	3
S1-57	62	0	0	0	0
S1-78	79	2	1	1	1

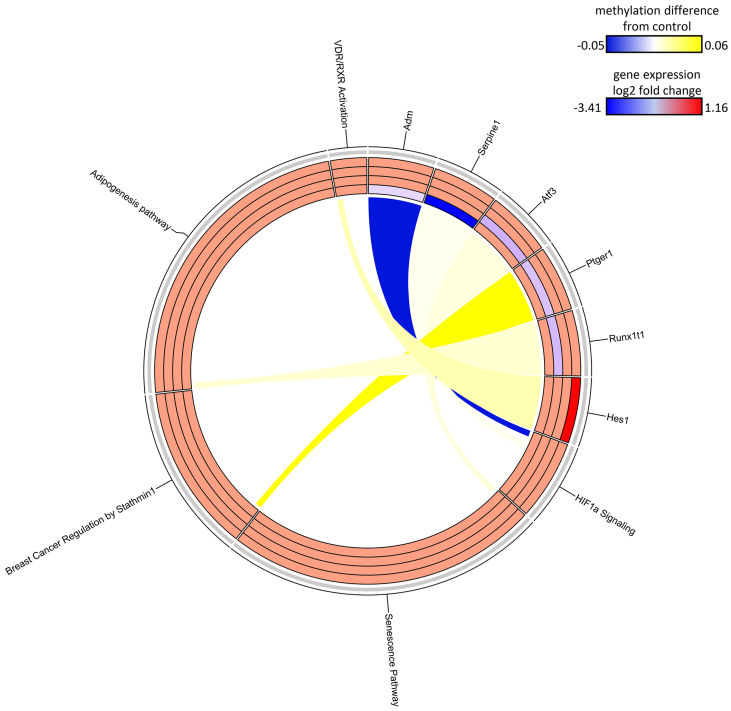

FIGURE 9
Circos plot of SDMR genes linked to "stop" subgroup gene pathways. Connections denote SDMR-linked genes with canonical gene pathways that are enriched by DEGs identified in the S1 groups. Color gradient of connections denote degree of methylation change compared to control mice (yellow = hypermethylated, purple = hypomethylated). Tracks denote $\log_2$ fold-change (red = upregulation, blue = downregulation) of gene expression compared to time-matched controls for S1 10-week, S1 32-week, S1 57-week and S1 78-week experimental groups (inside -> outside). Slice size for individual pathways is proportional to number of DEGs that link to each individual pathway.

release (35–37). Because of this metabolic reprogramming that appears to counteract the Warburg effect of cancer cells, DCA and other small molecule PDK inhibitors have been extensively studied as a cancer (and other metabolic disease) therapeutic (15). However, persistent effects due to early-in-life exposure to DCA instead *increased* tumor incidence compared to controls in mouse liver (1, 21–23) and the causal mechanism has not been fully characterized. Using gene expression and DNA methylation profiles of archived samples from a previously characterized stop exposure time-course study (22, 23), we further examined the underlying mechanisms of continuous and carryover effects of DCA in mouse liver. First, we observed significant gene expression changes linked to metabolic reprogramming with continuous DCA exposure; however, these expected patterns changed with age and transitioned to a pro-tumorigenic characterization. Second, carryover effects of DCA followed a different trajectory than continuous, 78-week DCA exposure, but also was heavily influenced by age. Finally, DNA methylation alterations measured at 78-weeks indicated both continuous and carryover effects of DCA exposure.

The tumorigenicity of DCA in rodent liver has been studied for nearly 30 years and evidence has stipulated that continuous higher exposures (*e.g.*, ≥3.5g/L in drinking water) can lead to overt hepatoxicity via disruption of normal cellular metabolism (9, 13) which could have a relationship with cellular regeneration but, there has not been a clear consensus on carcinogenic mode-of-action for direct DCA exposure (38). Our gene expression analysis recapitulated previous transcriptomic findings in many cases; the inclusion of time-matched controls over all measured time points also supported progression of phenotypic observations. By 32 weeks on continuous DCA exposure, gene expression alterations predictively enriched pathways involving activation of oxidative phosphorylation (*TCA cycle II*, *methionine degradation*), increased apoptosis signaling (*p53*, *ATM, death receptor*, *MYC signaling*), and decreased cell growth signaling and increased quiescence (*TGF-β*, *mTOR/AMPK*, *Id1*). We also observed upregulation of many Serpin family members (*e.g.*, *SerpinA3*, *SerpinD1*) and the Complement system (*e.g.*, *C9*) (39, 40) after 10 weeks of continuous DCA exposure that subsequently fell below control levels by 78 weeks, which matched the transient mild to moderate inflammation with early exposure (23). Interestingly, at the later timepoints of continuous DCA exposure, we observed increased proliferative signaling such as the *D-myo-inositol (1,4,5)-trisphosphate biosynthesis* pathway at 57-weeks and augmentation of the peroxisome proliferator-activated receptor alpha pathway (PPARαby 78-weeks, which flipped from a suppression of the PPARα pathway at 10-weeks of DCA exposure (Supplementary Figure 1). This activation was matched with inhibited STAT5b signaling, which has been noted mutually antagonistic to PPAR and other xenobiotic receptor activation and is characterized by "feminization" of the liver due to decreased growth hormone (GH) secretion leading to a profile more common in female

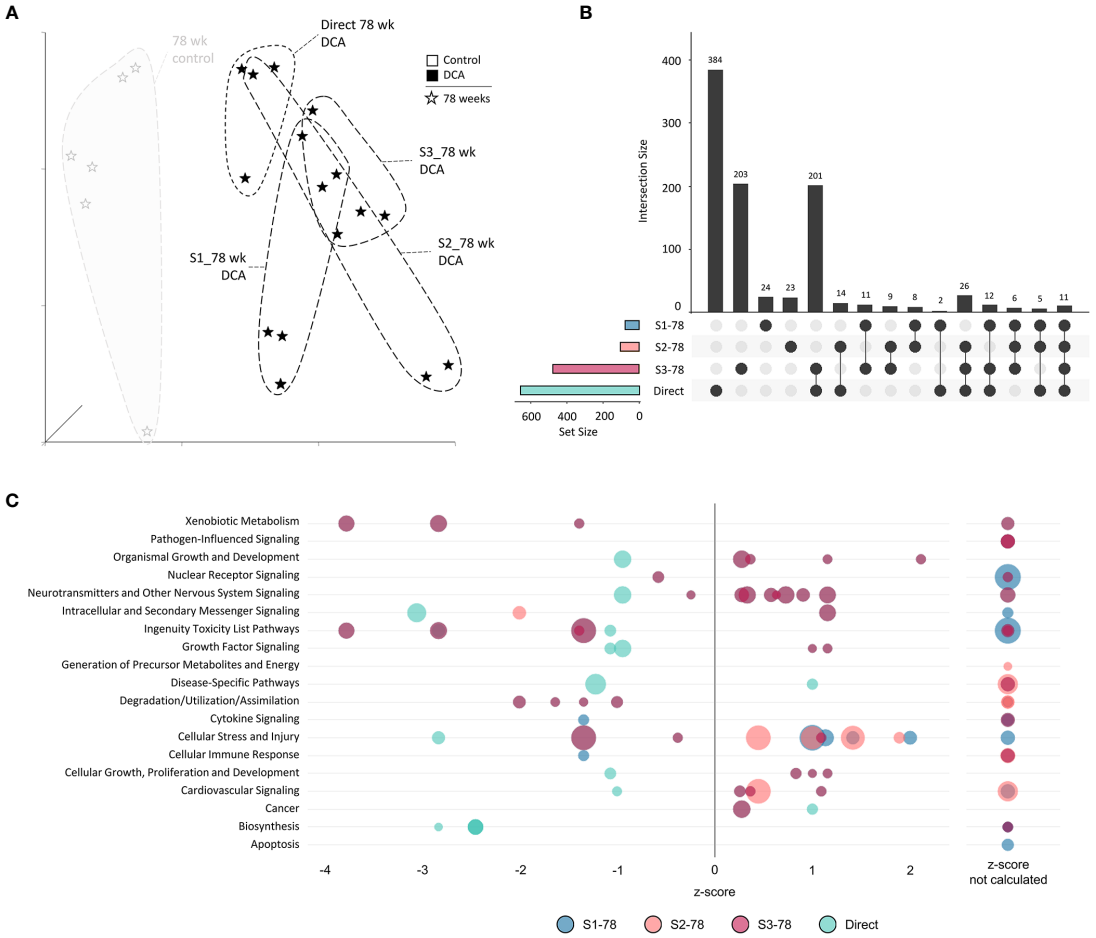

FIGURE 10
78-week subgroup differentially expressed genes and enriched canonical gene pathways. **(A)** Uniform Manifold Approximation and Projection (UMAP) for "78-week" subgroup samples. UMAP is based on 15 Principal Components of normalized count data before DEG determination, which captured over 99% of the variation displayed in these samples. **(B)** UpSet plot demonstrating differences and similarities of significantly (FDR-corrected p-value<0.05) altered genes compared to time-matched controls. Dots identify the group(s) that match to the adjacent bar and DEG number unique for those samples. **(C)** Bubble graph representing gene expression pathways enriched by the "78-week" subroup. Each bubble represents an individual canonical pathway, calculated by Ingenuity Pathway Analysis (IPA), that are categorized by larger meta-categories along the y-axis. The right-to-left placement identifies predicted pathway activation or repression (positive or negative z-score, respectively). Some pathways did not have the data to generate this prediction (no z-score). The size represents the -log(p-value) of the pathway enrichment. The color indicates the experimental mouse group that the pathway enrichment was measured.

TABLE 4 78-week groups DEGs and DDMR/SDMR-linked genes intersection.

		TSS-only Defined		TSS and Reg Region Defined		
	DEGs	DEG/DMR Gene Intersect	DEG/DMR Intersected Genes in Pathways	DEG/DMR Gene Intersect	DEG/DMR Intersected Genes in Pathways	
S1-78	79	14	10	7	6	DDMR
S2-78	102	14	7	9	5	
S3-78	476	41	17	24	11	
Direct	655	53	8	28	4	
S1-78	79	2	1	1	1	SDMR
S2-78	102	1	0	1	0	
S3-78	476	7	2	5	1	
Direct	655	9	0	5	0	

versus male rodents (41). GH secretion can be suppressed through an increased amount of circulating glucose levels such as during periods of increased gluconeogenesis (42); the converse is seen with high-dose DCA exposure (43). While we observed robust PPARα target gene induction previously with DCA treatment after 6 days (23), general consensus is DCA does not lead to liver cancer through a PPAR-mediated pathway (44). However, increased hepatic peroxisome proliferation activity was observed in mice with the dose used in this study, which equated to ~429 mg/kg-day (13, 23). This overall shift away from early DCA-like responses may reflect later processes initiated by early tumor formation at 78 weeks, which may be a result of DCA-induced hepatotoxicity with constant exposure, leading to secondary effects of regenerative proliferation and/or receptor-mediated mitogenesis.

DCA augments tumorigenesis even if the exposure window is limited to early life. Greater than 85% of the tumorigenic effect is captured with 10-weeks of exposure compared to a lifetime exposure at the same 3.5 g/L dose (22). The mechanisms of these persistent effects of DCA after short-term exposure are more uncertain than those of continuous exposure. Phenotypically, the most lingering effects in the liver included increased liver weight and hepatocellular hypertrophy, which resolved by 26 weeks after DCA removal. Liver weight again increased later in the study at 93 weeks likely due to the presence of tumors. Hepatocellular necrosis and mild inflammation also transiently persisted but resolved more quickly by 5 weeks after DCA removal (23). The effects were attributed to increased glycogenosis and glycogen storage which is an established hallmark in hepatocytes with high DCA exposure (9, 43) and may have contributed to acute hepatotoxic response during the exposure period. In our gene expression measurements, the two timepoints assessed after DCA removal (S1-32 and S3-78 weeks) exhibited a transcriptional burst of activity, with 155 DEGs in common, suggesting a similar response to the removal and return to homeostasis after DCA removal. Intriguingly, 70% of these genes exhibited opposite regulation between the two points, where the earlier 32-week timepoint favored known DCA effects of increased apoptosis, inflammation and suppressed growth signaling but, the converse was observed at 78-weeks. This is consistent with the phenotypic effects we previously measured, where mild necrosis and some inflammation, were observed at earlier ages with current DCA exposure, but were not observed with DCA exposure later in life (≥78 weeks) with current exposure (23). This suggested that the aged mouse liver responds differently to the effects of DCA. To further support that age heavily influenced cellular effects of direct or immediate post-DCA exposure, we observed similar overlap of 281 DEGs between the direct DCA exposed mice (S2-32) and recently exposed (S1-32), both samples of which were taken at 32 weeks. All but one of these genes shared the same directionality compared to controls and, again, linked to hallmark responses of DCA exposure. Therefore, immediate transcriptional carryover effects of DCA were robust, but mouse age heavily influenced the type of response much like what we observed in the direct only exposures: from DCA-like early to more cancer-like later.

Our results showed the transcriptional response to DCA in groups furthest out from DCA exposure (e.g., S1-78, S2-78) were greatly reduced compared to those mice closer to DCA exposure windows, but some signal did persist to the 78-week timepoint. The DEGs and gene pathways were similar between these two groups (100% of the shared DEGs displayed the same directionality) and were well represented by upregulated collagen genes that enriched pathways of apelin signaling and mechanisms that regulate fibrogenesis. Apelin is an adipokine that is the ligand for the G protein-couple receptor APJ (45) and has been of focus due to its sensitivity to glucose homeostasis and link to diabetes and insulin resistance (46). In liver, apelin exposure also promotes glycogenosis through the insulin signaling pathway (47), suggesting this response may be latent activity of DCA although hallmarks of excessive hepatic glycogen storage are no longer present at this point (23). Curiously, apelin is linked to liver fibrogenesis and mediates the expression of mesenchymal markers such as collagen (48–50), which are upregulated gene features at 78 weeks in the samples >26 weeks removed from DCA exposure. Conversely, a DCA derivative was linked to anti-fibrotic activity in hepatic stellate cells (HSCs) by suppressing the mesenchymal transition of these cells, presumably through anti-glycolytic effects of this compound (51, 52). This reflected the anti-fibrotic gene signature noted at 10-weeks of DCA exposure in our study (*e.g.*, reduced *HIF1α* pathway, reduced collagen gene expression). The persistent effects of DCA, which were more similar to results we observed with continuous DCA exposure later in life, reflected cellular processes that opposed the known effects of early, acute DCA exposure. This pattern, again, underscores an important interaction of DCA-mediated effects in the mouse liver and aging.

Commonality between the continuous and persistent effects of DCA exposure may be reflected in the DNA methylation profiles. Like gene expression, the methylation differences in the mice with 10-week, early-life DCA exposure were less robust than mice with continuous exposure by 78-weeks. Despite these differences, the altered methylation linked to gene pathways such as cellular signaling, regulation and metabolism shared in both the continuously exposed and the Stop groups. For example, we saw enrichment of genes linked to the gamma-aminobutyric acid (GABA) and glutaminergic signaling pathways in this intersection. The methylation profiles of these specific genes matched in the Direct and Stop samples, despite the general trend of hypomethylation in the Direct samples (also noted previously in female B6C3F1 mice [53)] and hypermethylation in the Stop samples. While commonly known as a neurotransmitter in the brain, GABA (a glutamate metabolite), can have effects in other metabolic organs including the liver (54). Hepatic GABA signaling is involved in mechanisms such as glucoregulation via insulin signaling and membrane transport, oxidative stress damage, and lipid metabolism (55–57). Gene pathways altered in our study that reflected such mechanisms were found in continuous DCA exposures (*e.g.*, *Nrf2 oxidative stress*), persistent effects (*e.g.*, *insulin pathway signaling*), and both (*e.g.*, *cholesterol biosynthesis*),

and were modulated by age and exposure length. Since DCA exposure increases glutamate production (58) and GABA-transaminase activity is increased through the TCA cycle which mediates GABA synthesis (54), likely there is an increase in GABA availability after early DCA exposure, impacting a variety of pathways, and leaving an indelible mark in the methylome.

We observed that genes linked to methylation changes after continuous exposure primarily overlapped DEGs at early time points for both the continuous and short-term DCA exposed mice, suggesting the aged methylome may reflect earlier events of DCA effects. The interrogation of methylation changes in the epigenome is increasingly seen in the context of lifetime and transgenerational assessments in human health. The seminal studies in the agouti mouse model demonstrated that prenatal nutritional supplements altered DNA methylation in the offspring with lasting phenotypic results into adulthood (59, 60). Similarly, the 2008 "Dutch Hunger Winter" study demonstrated that pregnancy during famine conditions led to increased adverse health outcomes in adult offspring, due to the methylation change in a single locus (61). Since that time, many environmental exposures have been examined in human and model population studies for methylation and disease state correlations (62, 63). Ongoing research in the field has reinforced the correlation between early life exposure and later life disease progression. Trevino et al. used a rat model study with an early life endocrine disrupting chemical and showed that early life epigenomic reprogramming led to adult metabolic disruption which was not evident until a later life dietary change (64). Li et al. examined lifetime methylation changes in a twin study assessing various physiological and health related parameters (65). They demonstrated that methylation changes in the epigenome, while most highly linked by genetic factors and gene regulation, are also strongly linked to cohabitation status, indicating the lifetime influence of early life environmental factors in adult growth, development, and likely disease progression. These methylation changes can either be persistent or transient once the environmental stressor is removed, leading to biomarkers that can indicate past or cumulative exposure or the "exposome" (66, 67).

Conclusion

Here, we measured the transcriptomic and epigenomic alterations due to increasing windows of exposure of the metabolic reprogramming chemical DCA in the male B6C3F1 mouse model. Previous studies were inconclusive about the mechanism of latent tumorigenic effects of DCA, not supporting classical routes such as genotoxicity, chronic oxidative stress, regenerative proliferation, and cytotoxicity. Using modern methods to assess 'omic-based measurements in archived formalin-fixed mice liver samples, we observed that responses due to DCA exposures early in life differed greatly than those later in life. In general, anticipated anti-Warburg related DCA effects were measured early, while opposing, more pro-tumorigenic pathways were noted later in life. Additionally, the length of time of DCA exposure impacted the robustness of response, but still followed a cancer-like "switch" which was dependent on mouse age. DNA methylation patterns at 78-weeks reflected early-life alterations in genes and pathways and likely impacted regulatory effects later in life. These data, along with previous studies, suggests that persistent metabolic shifts, whether they be due to consistent DCA exposure or persist through epigenetic means, interact with normal aging mechanisms to result in pro-tumorigenic environment. The impact of early-life, non-genotoxic exposures on later cancer outcomes is a major challenge for risk assessment when considering protective thresholds in the environment. By deriving the mechanistic basis for a chemical mode-of-action for latent carcinogenic effects in model systems, additional weight of evidence can be factored for transient exposures rather than relying on chronic life-time exposures in standard bioassays (1). In addition, biomarkers derived from these studies can be utilized to assist with chemical hazard identification where there is limited information, or where more non-classical tumorigenic modes-of-action, such as epigenetics, are occurring.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material and underlying data for all figures can be accessed at DOI: 10.23719/1529544. Sequencing data is available NCBI/Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) under accession #GSE242665. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by EPA Institutional Animal Care and Use Committee. This study used archived samples from this original study. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

GC: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. JC: Data curation, Formal analysis, Methodology, Software, Writing – original draft, Writing – review & editing. BB: Data curation, Formal analysis, Methodology, Software, Writing – original draft, Writing – review & editing. PB: Formal analysis, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing. BC: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration,

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2024.1389634/full#supplementary-material

SUPPLEMENTAL DATA 1
Complete DEG lists for all sample groups.

SUPPLEMENTAL DATA 2
IPA Canonical Pathway Analyses for DEGs in all sample groups.

SUPPLEMENTAL DATA 3
DMR locations and annotations for both Direct and Stop groups at 78 weeks.

SUPPLEMENTAL DATA 4
IPA Canonical Pathway Analyses of Genes Linked to DMR Regions Defined by Closest Transcriptional Start Site (TSS) for Both Direct (DDMR) and Stop (SDMR) Groups.

SUPPLEMENTAL DATA 5
DMR-linked Genes Defined by Closest Transcriptional Start Site (TSS) for Both Direct (DDMR) and Stop (SDMR) Groups that Intersect DEGs and Associated Canonical Pathways for All Timepoints.

SUPPLEMENTARY FIGURE 1
Ingenuity Pathway Analysis (IPA) *de novo* gene network generation based on DEGs from continuous 3.5 g/L DCA exposure in drinking water at 10 and 78 weeks. Arrows and capped lines represent known upregulation and downregulation of gene targets, respectively (orange = predicted upregulation matched experimental observation, blue = predicted downregulation matched experimental observation, yellow = predicted regulation did not match experimental observation, grey = IPA did not predict target regulation). Red and green shades represent upregulated and downregulated DEGs for that sample group, respectively, when greater intensity of those shades is proportional to greater fold-change. Shapes in figure represent different molecule types that translate/result from the expressed genes (rectangle = ligand-dependent nuclear receptor, square = cytokine, vertical diamond = enzyme, horizontal diamond = peptidase, horizontal oval = transcriptional regulator, vertical oval = transmembrane receptor, isosceles trapezoid = transporter, triangle = kinase, circle = other). **(A)** IPA de novo network for the S1-10 timepoint. PPARa and STAT5b-mediated transcriptional activity are predicted to be repressed (blue) and activated (orange), respectively. **(B)** IPA *de novo* network for the Direct (78-weeks) timepoint. PPARa and STAT5b-mediated transcriptional activity are predicted to be activated (orange) and repressed (blue), respectively.

SUPPLEMENTARY FIGURE 2
Chromosomal location of DMRs for Stop (S1-78) and Direct groups. For each mouse chromosome, the top figure represents the Stop group, while the bottom represents the Direct group. The *y-axis* represents the difference in methylation compared to control samples (drinking water only) at 78-weeks, where red dots are chromosomal locations of hypermethylated DMRs and blue dots are chromosomal locations of hypomethylated DMRs. Averaged intervals of hyper- or hypomethylation are represented by a gradient scale of red-to-blue, respectively, on the chromosome map (black colored internal represent no DMRs in that interval).

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Multiplicity of benign breast disease lesions and breast cancer risk in African American women

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The risk of developing subsequent breast cancer is higher in women diagnosed with benign breast disease (BBD) but these studies were primarily performed in non-Hispanic white populations. Still, these estimates have been used to inform breast cancer risk models that are being used clinically across all racial and ethnic groups. Given the high breast cancer mortality rates among African American (AA) women, it is critical to study BBD in this population, to ensure the risk models that include this information perform adequately. This study utilized data from AA women who underwent benign breast biopsies at a hospital served by the University Pathology Group in Detroit, Michigan, from 1998 to 2010. Patients were followed for subsequent breast cancers through the population-based Metropolitan Detroit Cancer Surveillance System (MDCSS). BBD lesion scores were assigned to represent the severity or extent of benign breast lesions, with higher scores indicating a greater number of distinct lesion types. Of 3,461 eligible AA women with BBD in the cohort, 6.88% (n=238) subsequently developed breast cancer. Examined individually, six of the eleven lesions (apocrine metaplasia, ductal hyperplasia, lobular hyperplasia, intraductal papilloma, sclerosing adenosis, columnar alterations and radial scars) were significantly associated with increased risk of breast cancer after adjustment for age and year of biopsy and were further considered in multiple lesion models. For every different type of benign breast lesion, subsequent risk of breast cancer increased by 25% (RR=1.25, 95% CI: 1.10, 1.42) after adjustment for age at biopsy and proliferative versus non-proliferative disease. In summary, this study affirms the increased breast cancer risk in AA women with BBD, particularly in those with multiple lesions. These findings have implications for the management of breast cancer risk in millions of women affected by BBD, a high risk group that could benefit from personalized surveillance and risk reduction strategies.

KEYWORDS

African American, breast cancer, benign breast disease, breast biopsy, hyperplasia

Introduction

In the United States, African American (AA) women have the highest breast cancer (BC) mortality rates compared to other racial and ethnic groups. Additionally, the incidence rates among AA women are also higher than all other racial and ethnic groups, other than non-Hispanic white women (1). First described by Dupont et al. in 1980, women diagnosed with benign breast disease (BBD) have a higher risk of subsequent development of BC (2). Based on this seminal work, BBD lesions are often categorized into three groups: non-proliferative disease (NPD), proliferative disease without atypia (PDWA), and atypical hyperplasia (AH). Of these, AH is associated with the greatest subsequent risk of BC, with approximately 1 in 4 women developing a subsequent BC over the next two decades (3). AH has been identified in up to 10% of BBD biopsies, and randomized, controlled trials with existing cancer prevention therapies have shown a substantial risk reduction benefit (4). PDWA, representing ~40% of all BBD, is associated with at least a 2-fold increase in BC risk. Even biopsies that show NPD, the lesions with lowest risk, were still associated with risk that is 25% greater than that of women who have never undergone a clinical biopsy. As reviewed by Dyrstad et al, women with proliferative types of benign breast disease, both with and without atypia, have increased risk of BC (5).

Despite several decades of research, the studies that have examined BBD have been primarily comprised of non-Hispanic white women (3, 6–8). Given the earlier onset of disease and the poorer prognosis experienced by AA women, it is critical to better define the risk of BBD lesions and subsequent risk of BC in this population. Here, we assess the association between various types of BBD lesions and subsequent BC, quantify the BC risk associated with multiplicity of these lesions, and provide a description of the types of BC that have developed in this high-risk cohort of AA women with BBD.

Methods

Study population

The Detroit Benign Breast Disease Cohort (BBD Cohort) is comprised of n=3,860 AA women (self-reported race obtained via medical record abstraction) who had a benign breast biopsy at a hospital served by the University Pathology Group from 1997-2010. Patients were followed for subsequent BCs, defined as an invasive or *in situ* cancer that occurred at least 6 months after the date of the benign biopsy, through the Metropolitan Detroit Cancer Surveillance System (MDCSS), a founding site of the National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) program. The last linkage to MDCSS was May 19, 2022; so, for participants who did not have diagnosis of BC or death, this was the date of censor for all analyses. Cote et al. provided more details regarding the study design (9). This study was approved by the Wayne State University Institutional Review Board (#087812M1E).

All the hematoxylin and eosin (H & E)-stained benign biopsy slides from each case (ranging from 2-22 slides per case) were retrieved from the Department of Pathology at Wayne State University and assessed for 11 different benign lesions by the study pathologist (RAF): apocrine metaplasia, ductal hyperplasia, lobular hyperplasia, calcifications, cysts, duct ectasia, fibrosis, intraductal papilloma, sclerosing adenosis, columnar alterations, and radial scar. Using these markers, the study pathologist also categorized the case using the criteria established by Dupont and Page: non-proliferative disease, proliferative disease without atypia, and proliferative disease with atypia (atypical hyperplasia). For the purposes of this analysis, women with atypical hyperplasia were removed from the study population (n=149), as there is a known, strong association with risk of subsequent BC (3). In addition, 250 cases were excluded that had slides containing only a large fibroadenoma where additional BBD features could not be assessed. Therefore, the dataset used in this analysis included a total of 3,461 participants.

Laboratory analysis

To confirm the BC subtype listed in the original diagnostic pathology reports, we utilized formalin fixed, paraffin-embedded tumor blocks from a subset of women (n=100) identified with a subsequent cancer to examine the following markers: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Briefly, the slides were deparaffinized and rehydrated. Antigen retrieval was performed, followed by immunohistochemical staining using a Ventana automated immunohistochemical stainer, counterstained (when necessary), dehydrated, and mounted. The pathologist was blinded to the clinical characteristics and prior pathology report associated with the tissue. All antibodies were sourced from DAKO, and the specific conditions and positivity assessment are as follows: ER, clone 1D5, 1:100 dilution, positive if greater than 1% of nuclei stained; PR, clone PgR636, 1:100 dilution, positive if greater than 1% of nuclei stained; HER2, Hercept Test (pre-diluted), greater than 30% of cells showed circumferential intense and uniform staining. There was approximately a 90% concordance between the prior reports and the repeated analysis (data not shown), thus the receptor data abstracted from the pathology reports was used.

Statistical analysis

This was a retrospective cohort study. Cases were classified as women who had a subsequent BC at least six months after their

Abbreviations: AA, African American; AH, Atypical Hyperplasia; BBD, Benign Breast Disease; BC, Breast Cancer; BCRAT, Breast Cancer Risk Assessment Tool; BRCA, Breast Cancer gene; CI, Confidence Interval; DCIS, Ductal carcinoma in situ; ER, Estrogen Hormone Receptor; H & E, Hematoxylin and Eosin; HR+, Hormone receptor positive; HER2-, Hormone Estrogen Receptor 2 negative; IBIS Model, International Breast Intervention Study model; MDCSS, Metropolitan Detroit Cancer Surveillance System; NPD, Non-proliferative disease; PR, Progesterone Hormone Receptor; PDWA, Proliferative disease without atypia; RR, Relative Risk; SEER, Surveillance, Epidemiology and End Results; TNBC, Triple Negative Breast Cancer.

initial biopsy. Follow up time in months was calculated from date of biopsy to the date of BC diagnosis or death, whichever occurred first. Women were classified as controls if there was no record of a subsequent BC in MDCSS, and their follow up time was calculated from date of biopsy to: 1) death from other cause or 2) date of last cohort-MDCSS linkage.

Baseline characteristics were compared between cases and controls with chi-square tests for categorical variables and Wilcoxon's rank sum test for continuous variables. The relative risk (RR) for each marker was estimated with logistic regression, adjusted for age and year of biopsy. A score was created by summarizing the number of markers which had univariate logistic regression p<0.05: ductal hyperplasia, lobular hyperplasia, intra-ductal papilloma, sclerosing adenosis, columnar alterations, and radial scars. The score ranged from 0 to 6, with 0 representing "no lesions" and 6 meaning that all 6 distinct types of lesions were present. All statistical tests were two sided, but p- values should be interpreted with caution due to issues of multiplicity testing. The widths of the 95% confidence intervals were not adjusted for multiplicity testing and cannot be used in place of a hypothesis test. All statistical analyses were performed using SAS software version 9.4 (Cary, NC).

Results

Among the 3,461 eligible AA women with BBD in our study, 238 women subsequently developed BC. As shown in Table 1, those who developed BC were slightly older than those who did not (51 years versus 47 years at initial benign biopsy) and were more likely to have proliferative disease without atypia compared to non-proliferative lesions (p=0.001).

The distribution of 11 different types of benign breast diseases is shown in Table 2, along with the risk of developing BC associated with each type of lesion, adjusted for age and the year of the initial biopsy. There were six lesions that were associated with increased risk of subsequent BC that were used to create the score variable described in Table 3: ductal hyperplasia, lobular hyperplasia, intra-ductal papilloma, sclerosing adenosis, columnar alterations and radial scars. Overall, pathologic classification of proliferative disease without atypia was associated with a 57% increase in risk of developing a subsequent cancer compared to those biopsies classified as non-proliferative, but this composite variable was not included in the score variable classification.

Table 3 describes the distribution of number of BBD lesions which is reported as both a continuous and categorial variable. The mean score for cases was higher compared to controls (1.7 and 1.2, respectively, p-value <0.001).

Table 4 depicts the risk of subsequent BC by benign breast lesion score, adjusted for age at biopsy and proliferative or non-proliferative disease. Each additional feature was associated with a 25% higher relative risk of developing BC (RR 1.25, 95% CI 1.10-1.42, p-value 0.001) after adjustment. The BBD lesion score demonstrated its independent predictive role. Additionally, each

TABLE 1 Characteristics of women with benign breast disease by case/control status in the Detroit BBD Cohort.

Variable	Control (n=3,223)	Case (n=238)	p value
Age at Biopsy– median years (range)	47 (18,84)	51 (26,81)	**<0.001**
Follow up – median years (range)	16.9 (0.5,23.0)	8.9 (0.7,23.4)	**<0.001**
Biopsy year - no. (%)			0.473
1997-2000	1,131 (35)	89 (37)	
2001-2010	2,092 (65)	149 (63)	
Dupont and Page Classification - no. (%)			**0.001**
Non-Proliferative Disease	1,889 (59)	114 (48)	
Proliferative Disease without Atypia	1,334 (41)	124 (52)	

Bold values indicate statistical significance at the p < 0.05 level.

TABLE 2 Distribution of type of benign lesion and risk of subsequent breast cancer in the Detroit BBD Cohort.

Variable	Control		Case			
	N	%	N	%	RR*	(95% CI)
Total	3,223		238			
Apocrine Metaplasia					1.06	0.80-1.40
No	2,250	70%	159	67%		
Yes	973	30%	79	33%		
Ductal Hyperplasia					**1.39**	**1.05-1.84**
None	2,345	73%	153	64%		
Yes	878	27%	85	36%		
Lobular Hyperplasia					**10.96**	**2.90-41.41**
None	3,218	100%	234	98%		
Yes	5	0%	4	2%		
Calcifications					1.04	0.78-1.39
No	2,109	65%	139	58%		
Yes	1,114	35%	99	42%		
Cyst					1.19	0.91-1.55
No	2,005	62%	135	57%		
Yes	1,218	38%	103	43%		
Duct Ectasia					1.14	0.79-1.64
No	2,753	85%	201	84%		
Yes	470	15%	37	16%		
Fibrosis					1.13	0.86-1.48
No	1,485	46%	100	42%		
Yes	1,738	54%	138	58%		

(Continued)

TABLE 2 Continued

Variable	Control		Case			
	N	%	N	%	RR*	(95% CI)
Intra-Ductal Papilloma					**1.61**	**1.13-2.28**
None	2,861	89%	195	82%		
1 or more	362	11%	43	18%		
Sclerosing adenosis					**1.80**	**1.34-2.41**
No	2,450	76%	160	67%		
Yes	773	24%	78	33%		
Columnar alterations					**1.86**	**1.41-2.46**
No	2,436	76%	148	62%		
Yes	787	24%	90	38%		
Radial Scar					**2.13**	**1.11-4.12**
No	3,157	98%	227	95%		
Yes	66	2%	11	5%		
Proliferative Disease						
No	1,889	59%	114	48%	**1.57**	**1.20-2.05**
Yes	1,334	41%	124	52%		

*adjusted for age and year of biopsy.
Bold values indicate statistical significance at the p < 0.05 level.

one-year increase in age at biopsy was associated with a 3% higher relative risk (RR) of developing a BC (RR 1.03, 95% CI 1.02-1.03, p-value <0.001).

Table 5 provides information on the subsequent BC subtype. Out of the total BC cases, 166 cases (69.7%) were invasive, with hormone receptor positive, HER2 negative cancers the most

TABLE 3 Distribution of number of lesions (scores) by case/control status, the Detroit BBD Cohort.

	Control		Case		p value
Score as a continuous predictor					***<0.001***
Mean (std)	1.2	(1.3)	1.7	(1.4)	
Median (range)	1	(0,6)	1	(0,6)	
Score as a categorical predictor, N (%)					***<0.001***
Score 0 (no lesions)	1149	36%	56	24%	
Score 1	971	30%	65	27%	
Score 2	562	17%	49	21%	
Score 3	339	11%	38	16%	
Score 4	146	5%	19	8%	
Score 5	47	1%	9	4%	
Score 6	9	0%	2	1%	

Bold values indicate statistical significance at the p < 0.05 level.

TABLE 4 Multivariable logistic regression for the risk of subsequent breast cancer by benign breast lesion score, the Detroit BBD Cohort.

	RR (95% CI)		p value
BBD lesion Score	**1.25**	**1.10-1.42**	**0.001**
Dupont and Page Classification			0.956
Non-Proliferative Disease	Ref.		
Proliferative Disease without Atypia	1.01	0.71-1.45	
Biopsy age (per 1 year)	**1.03**	**1.02-1.04**	**<.001**

Bold values indicate statistical significance at the p < 0.05 level.

frequent subtype (55.4%). Triple negative BC, known to be more frequently diagnosed in AA women, comprised 19.0% of invasive cancers in this cohort. For 24 cases (14.5%), subtype could not be determined (insufficient data or tissue available).

Discussion

In this cohort of nearly 4,000 AA women who had undergone a clinically indicated breast biopsy resulting in a benign diagnosis, we report an increased risk of subsequent BC with several types of benign breast lesions, specifically ductal and lobular hyperplasia, intraductal papilloma, sclerosing adenosis, columnar alterations and radial scars. These observations are similar to those in primarily non-Hispanic white populations, as reviewed by Dyrstad et al. Further, and never examined in a cohort comprised of only AA women, is the finding that as the individual types of BBD lesions found within a single breast biopsy increases, subsequent risk of BC also rises. Multiple types of BBD in a single biopsy are common, with 38% of women included in the National Surgical Adjuvant Breast and Bowel Project's BC Prevention Trial having more than one type of lesion, similar to the 35.2% reported in our study population (10). Our results are also consistent with those of Worsham and colleagues, who studied the multiplicity of concurrent BBD lesions in another population of women with BBD of similar size from metropolitan Detroit, which was racially diverse (28% were AA). Multiple NPD lesions in a single biopsy were associated with increased risk of BC (RR=1.79, 95% CI: 1.0, 3.21) as were women with multiple PDWA, with a 2.87-fold risk of BC (RR, 2.87; 95% CI, 1.70-4.83) compared to

TABLE 5 Subtypes of Breast Cancers in the Detroit BBD Cohort.

Subtype	Number (%)
In Situ	72 (30.3%)
Invasive	166 (69.7%)
HR+, HER2-	92 (64.8%)
HR+, HER2+	15 (10.6%)
HR-, HER2+	8 (5.6%)
TNBC	27 (19.0%)

Note: 24 invasive tumors could not be analyzed, thus the denominator for the subtype analyses is n=142.

women with only one NPD lesion (11). Importantly, they found that the effects of multiple lesions did not differ by race. More recently, Sherman et al. examined subsequent risk among women who received a percutaneous biopsy with benign findings and considered multiple lesions within the Dupont and Page classification system. They noted a higher risk for both non-proliferative lesions (3 or greater, HR=1.47, 95% CI: 1.14-1.88) as well as proliferative lesions without atypia (3 or greater, HR=2.14, 95% CI: 1.29-3.53) (12). As the majority of breast biopsies are now percutaneous versus surgical excisions, determining whether risk is similar regardless of biopsy type is critical when considering how informative this variable is when modeling BC risk.

At least two BC risk assessment models, the Breast Cancer Risk Assessment Tool (BCRAT, also known as a modified version of the Gail model) and the International Breast Intervention Study model (IBIS, or the Tyrer-Cuzick model) utilize information regarding personal history of benign breast biopsies (13, 14). Only one model has been developed specifically for women with BBD, which extended the BCRAT tool by incorporating detailed pathologic characterization of the benign lesions seen on biopsy, including a number of specific benign lesions as well as overall histologic impression (proliferative vs. non-proliferative) (15). External validation of this model has been limited as most studies do not have access to this detailed pathologic information, thus this model has not been widely utilized. Furthermore, these models have been built and validated in populations of non-Hispanic white women and have lower discriminatory accuracy in other racial and ethnic groups (16, 17). Our findings regarding risk of BC in a group of AA women with BBD highlight the importance of the development of models that provide more concise estimates to inform prevention and screening strategies.

At 30%, the proportion of *in situ* BCs in this population was higher than what is reported in the general population (20% of all BCs) (18). The majority of *in situ* BCs are ductal carcinoma in situ, or DCIS, and the increase in incidence has been attributed to the rise in mammographic screening. As our population of women with BBD have some modality of breast screening, it is not surprising that our proportion of DCIS is higher than in the general population. Among women with invasive BC in this BBD cohort, the majority had ER/PR+, HER2- cancers, whereas 19.0% of invasive tumors were classified as triple negative BC (TNBC). The proportion of TNBC cases in our cohort is slightly lower than what has been reported among non-Hispanic black women nationally (25.6%) (19) and what was reported by Newman et al. in the only other BBD cohort that contains a large proportion of AA women (24.2%) but within the same range (20). While most risk models have grouped together all subtypes of BC, or examined hormone positive cancers, less work has focused on TNBC, a subtype that is more common in AA women regardless of a prior history of BBD.

Our study has notable strengths, as the only cohort focused on benign breast disease and subsequent BC risk in the AA population. Furthermore, all slides (compared to a subset) from the BBD biopsy were re-reviewed by a breast pathologist versus relying on pathology reports. As the risk associated with atypical hyperplasia has already been well-defined and usually requires further treatment or surveillance, we removed this group with the highest risk, allowing for the examination of other types of BBD lesions separate from this established risk, or interventions that may have lowered the risk of subsequent BCs. Additionally, the study encompassed a wide spectrum of BBD lesion types allowing the examination of BBD multiplicity. The cancers that developed were identified through a population-based cancer registry, which allowed access to pathologic details (i.e., receptor status) when tissue was unavailable for testing.

In addition to these strengths, our study also had limitations. As a retrospective cohort study, we did not contact the women included in the BBD cohort. Specifically, we do not have detailed information regarding other risk factors associated with BC, such as family history, BRCA1/BRAC2 status and other reproductive factors included in risk models. Thus, we do not have the ability to develop our own model or test other models in this BBD cohort. We also may have missed some BC cases if the study participant left the tri-county area covered by the cancer registry, although an analysis of Southeastern Michigan Census data from 2000 to 2010 suggested most population movement was within the tri-country area (data not shown). This potential information bias would result in moving the risk estimates towards the null. Additionally, these results, while obtaining cancer data from a population-based registry, are still from a single geographical area. Thus, the results may not be generalizable to other AA women in the United States or women of African ancestry residing elsewhere. Lastly, the methodology employed to formulate the lesion multiplicity assessment (i.e., the benign breast score) assumes uniform risk across all lesions, which is unlikely from a biological perspective.

In conclusion, our study affirms the increased BC risk in AA women with BBD, particularly in those with multiple lesions. There is a clear need to better characterize factors associated with risk in women with BBD. In 2019, the U.S. Preventive Services Task Force recommended the utilization of BC risk assessment models to offer BC chemoprevention in higher risk women (recommendation level B), and specifically highlighted those with BBD: "*…This recommendation applies to asymptomatic women 35 years and older, including women with previous benign breast lesions on biopsy…*" despite the paucity of research in women with BBD as related to the current risk models (21). Inclusion of diverse populations, particularly those which bear a disproportionate burden of disease, are critical to ensuring evidence-based research and prevention approaches benefit all.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Wayne State University Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because Retrospective tissue study exemption 4.

Author contributions

VPat: Writing – original draft. JR: Data curation, Formal analysis, Methodology, Writing – original draft. WC: Formal analysis, Writing – review & editing. JB: Data curation, Investigation, Methodology, Writing – review & editing. EA: Data curation, Investigation, Writing – review & editing. BA: Data curation, Investigation, Writing – review & editing. VPar: Data curation, Investigation, Writing – review & editing. AS: Data curation, Investigation, Methodology, Writing – review & editing. SB: Data curation, Investigation, Writing – review & editing. RA-F: Data curation, Methodology, Supervision, Writing – review & editing. MC: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Cell proliferation and carcinogenesis: an approach to screening for potential human carcinogens

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Cancer arises from multiple genetic errors occurring in a single stem cell (clonality). Every time DNA replicates, mistakes occur. Thus, agents can increase the risk of cancer either by directly damaging DNA (DNA-reactive carcinogens) or increasing the number of DNA replications (increased cell proliferation). Increased cell proliferation can be achieved either by direct mitogenesis or cytotoxicity with regenerative proliferation. Human carcinogens have a mode of action of DNA reactivity, immunomodulation (mostly immunosuppression), increased estrogenic activity (mitogenesis), or cytotoxicity and regeneration. By focusing on screening for these four effects utilizing *in silico*, *in vitro*, and short-term *in vivo* assays, a biologically based screening for human chemical carcinogens can be accomplished with greater predictivity than the traditional 2-year bioassay with considerably less cost, less time, and the use of fewer animals.

KEYWORDS

two-year bioassay, mutagenesis, immunosuppression, estrogenic activity, cytotoxicity, regenerative proliferation, cell proliferation

Introduction

Means to discover chemicals that can increase the risk of cancer in humans has been a goal of science for over a century. Approaches have involved the development of epidemiology studies of various populations, beginning originally with studies of various occupational settings but more recently utilizing investigations of broad populations. The second means of screening for carcinogens is to evaluate the chemicals in animal models, most notably the long-term rodent bioassays in rats and mice, which have evolved since the 1960s. Considerable concern has arisen over the years about the performance of such studies so that efforts are now underway to develop alternative tests to screen for chemical carcinogenicity. Furthermore, efforts are being made to reduce the number of animals utilized in toxicologic evaluations.

Concerns regarding the long-term rodent bioassays have extensively been described in the literature and include the high cost, the long time to perform such assays, the use of large numbers of animals, and the interpretation of the assays, but most notably there is increasing realization that the results of the long-term bioassay in rodents are frequently not predictive of effects in humans (1–5). Any time an experiment is performed in animal models, two basic assumptions are made, namely: (1) what happens in the animal model will also happen in humans (species extrapolation) and (2) the response observed at the doses used in the animal model will be relevant to the exposure levels in humans (dose extrapolation). For some chemicals, these assumptions may be reasonable; however, for many chemicals, one or both of these assumptions are incorrect.

Lack of relevance of the animal findings to humans includes such examples as α_2u-globulin as related to kidney tumors in male rats (6), PPARα activator-related liver tumors in rats and mice (7, 8), urinary bladder tumors secondary to the administration of high doses of various sodium salts (saccharin, ascorbate, and bicarbonate) inducing urinary bladder tumors in rats (6), and statins producing liver tumors in rats and mice (9–11). Numerous examples have likewise been identified of tumors being produced in animal models at a high dose that are not relevant to human exposures at lower levels, with a prototypic example being chloroform-induced liver and kidney toxicity and tumors (12). Humans exposed to high doses of chloroform also produce liver and kidney toxicity, but environmental exposures in the drinking water are at concentrations several orders of magnitude less and do not produce cytotoxicity. They are therefore not considered relevant to human cancer risk at human exposure levels. The presence of thresholds have been clearly demonstrated for non-genotoxic carcinogens, and there is increasing evidence that even for genotoxic carcinogens there is a threshold (13–17). Exposure to levels below these thresholds would not increase cancer risk.

There have been many attempts to identify alternative tests for screening for carcinogenicity for chemicals, beginning with the development of the Ames genotoxicity assay utilizing *Salmonella* bacteria (18, 19). Although numerous other examples have been developed for specific types of tumors, there remains no clear approach utilizing alternative methods to evaluate carcinogenicity that is accepted both scientifically and in regulatory settings.

Nevertheless, considerable effort is being made to develop alternative approaches. This includes the approach being taken by the pharmaceutical industry as illustrated in the new ICH (20) guidelines that have been developed to provide guidance for the kinds of data that can be used to provide a weight of evidence evaluation that would preclude the necessity of performing long-term bioassays. Waiver programs have likewise been developed in various agencies, including the US Environmental Protection Agency (21) and the European Chemical Agency (ECHA), attempting to reduce the reliance on animal testing for carcinogenicity. The focus of these new approaches is entirely based on the mode of action considerations.

The present approach to carcinogenicity screening utilizing animal models is to perform the long-term assay, identify any tumors that are increased in incidence in rats and/or mice, and then evaluate whether the mode of action and/or the dose is relevant to human exposures. A framework for the evaluation of mode of action and human relevance of toxic endpoints has been developed by the International Programme on Chemical Safety (IPCS) and by the US EPA and Health Canada (22–26). This has been incorporated into regulatory guidelines and has evolved to the development of adverse outcome pathways (AOP) (27, 28). The newer alternative approaches start with the idea of evaluating various modes of action in short-term *in vivo* and *in vitro* assays, with long-term animal testing not being required. Various approaches have been described based on the mode of action (4, 5), and this paper describes an elaboration of that approach.

Basic principles of carcinogenesis

Utilizing a mode of action approach, we first have to develop a basic understanding of carcinogenesis. Research over the past century has clearly demonstrated that cancer arises due to multiple genetic errors occurring in the stem cell population of a given tissue (4, 5, 29, 30). More than one error is required, although how many errors are actually required for individual tumors is generally not definitively known at this time. All of the errors in the DNA must accumulate in a single cell since cancer is considered a clonal disease. Furthermore, carcinogenesis is considered a probabilistic (stochastic) process, that is, it is not whether exposure to chemical X actually leads to tumor Y, but rather whether the level of exposure to X has a certain probability of leading to the development of tumor Y. In cancer, the mistakes have to occur in the genes that are critical to the development of a given cancer and have to be in a portion of that gene that is relevant to its function or control of its expression. Lastly, it is well known that every time DNA replicates those mistakes occur, albeit uncommonly; nevertheless, mistakes occur every time DNA replicates.

Based on these considerations, a chemical can increase the risk of cancer in one of two basic ways, namely: (1) the chemical can damage DNA directly so that more mistakes are made every time DNA replicates or (2) the agent can increase the number of DNA replications, providing more opportunities for critical mistakes to occur in the critical genes, leading to the development of cancer (29–31). These effects on toxicity or cell proliferation can be direct or indirect, frequently require metabolic activation of a chemical, and can be secondary to the activation of other systems such as immunomodulation, leading to the increased expression of various viruses which are known to be oncogenic (32, 33).

Numerous metabolic activation processes have been identified for DNA-reactive carcinogens, which have been well delineated in the literature. It is this process that is best screened for by the Ames Assay and other mutagenesis assays as well as computerized Structure Activity Relationships (SAR) (34).

Numerous processes have likewise been identified by which increased DNA replication can be produced (35, 36). It is important to recognize that this is the number of DNA replications and not necessarily the rate, although they frequently go together. However, there has been a misunderstanding in the literature that increased

cell proliferation is recognized only by an increase in rate, usually determined by labeling indices such as bromodeoxyuridine (BrdU) or Ki-67 immunohistochemical methods. This issue is particularly notable in the gastrointestinal tract where the stem cell population is already proliferating at a high rate, and increased proliferation is generally not reflected by an increase in rate but rather an accumulation of the appropriate stem cell population (such as the crypts of the intestine) (37).

Increased cell proliferation can be caused by either an increase in cell births or decrease in cell deaths, which leads to an accumulation of more cells (31, 38, 39). Increased cell births can be produced either by direct mitogenesis (directly inducing cells to replicate) or, more commonly, by cytotoxicity (cell death) with consequent regenerative proliferation. Decreased cell deaths can be produced either by increasing apoptosis in certain tissues or decreasing cell differentiation, which is a cell death process. More than one of these processes may be present for a given chemical. DNA-reactive carcinogens if administered at high enough doses will also produce cytotoxicity with regenerative proliferation, leading to a synergistic effect. This has been illustrated with the carcinogen 2-acetylaminofluorene (2-AAF) for the development of liver and urinary bladder tumors in mice in the so-called megamouse (ED_{01}) study performed at the National Center for Toxicological Research in the 1970s and modeled by Ellwein and Cohen in the 1990s showing the interaction between DNA damage and increased cell proliferation (40).

Modes of action of human carcinogens

Although numerous chemicals and other agents have been identified as causing cancer in humans, fundamentally they all act by one of four basic modes of action, namely: (1) DNA-reactive mutagenesis, (2) immunosuppression, (3) increased estrogenic activity, and (4) cytotoxicity and consequent regenerative increased cell proliferation. Multiple examples of these have been identified in the human population.

A variety of classes of mutagenic carcinogens have been identified in the past century, beginning with polycyclic aromatic hydrocarbons (PAH) and subsequently including such agents as aromatic amines, N-nitrosamines, aflatoxins, phosphoramide mustards, aristolochic acids, and other agents. Such chemicals are usually positive in the Ames Assay, particularly if an appropriate metabolic activating system is utilized (18, 19). It is important to note that just because a chemical produces a DNA adduct does not mean that it necessarily will be mutagenic and therefore not necessarily carcinogenic (13, 14). The adduct has to be at a site that is involved in base pairing or can be shown to produce apurinic or apyrimidinic sites. These chemicals are also usually positive in the long-term rodent bioassay.

Immunosuppression or, more accurately, immunomodulation is also well known to be the basis for human carcinogenesis (32, 33). Immunosuppression can be produced in humans either by an inherited disorder, secondary to treatment with pharmaceuticals used for transplantation or for the treatment of various diseases such as autoimmune disorders or various cancers, or by AIDS. Regardless of the cause of the immunosuppression, there is an increased risk for the development of cancer. However, it is not an increased risk of all cancers; rather, it is an increased risk mostly of tumors related to the activation of various oncogenic viruses such as Epstein–Barr virus (EBV), human papilloma virus (HPV), or Kaposi's sarcoma virus (KSV, also known has human herpes virus 8, HHV8) and possibly others. Also increased are certain other tumors such as melanoma, which has a high mutation rate and presumably generates neoantigens. Kidney transplant patients also develop an increased risk of kidney cancers due to their chronic kidney disease since kidneys usually are retained in the patient in whom the transplant is placed. Chronic kidney disease is a well-known cause of increased risk of renal tubular tumors (41). A major difficulty with the long-term rodent bioassay is the fact that many immunosuppressive agents are actually negative in that assay whereas they are well known to cause cancer in humans (42). Thus, the animal bioassay produces false negative results, the worst outcome for any screening assay. False positives can be dealt with, but false negatives are a serious issue.

Increased estrogenic activity is also the cause of certain tumors in humans, most notably breast cancer and endometrial cancer but also including uncommon liver hepatocellular tumors and possibly tumors at other sites (43). This is generally reflected as not just an increase in estrogen but also an imbalance between estrogen and progesterone. Estrogen-related carcinogenesis is related to the mitogenic effect of the interaction of estrogen and estrogen-like chemicals with estrogen receptors, but there is evidence that for at least the liver and possibly the breast, those DNA adducts that form from estrogen metabolites might also play a role (44). Estrogen and estrogenic chemicals also produce tumors in animal models, but dose is a critical consideration.

Lastly, there is the mode of action of cytotoxicity and consequent regeneration. This is a common mode of action in animal models for non-genotoxic chemicals, such as chloroform as mentioned above (12), but has been clearly associated with chemicals in humans (4). Arsenic is one example (45). This mode of action clearly shows a threshold as part of the dose response, and frequently the dose is quite high in animals compared to exposures in humans, with the example being chloroform again.

Screening for human carcinogens

Since human carcinogens act by one or more of these four modes of action, screening for human carcinogens can be based on screening for these four modes of action, much of which can be accomplished with assays that are already available, many of which are *in vitro* but not all (4, 5).

Screening for mutagenesis is the most developed of the four modes of action. This involves essentially performance of the Ames Assay with appropriate metabolic activating systems (18, 19). Chemicals involving indirect genotoxicity, such as chromosomal aberrations and micronucleus formation, are not strictly appropriate for this determination. To begin with, these latter assays rely on cytotoxicity for the results, are indirect, and involve

thresholds (46–49). Furthermore, it is not clear that these assays are strictly predictive of carcinogenicity rather than indirect genotoxicity being related to cytotoxicity. Computational models for mutagenicity are well developed and can be utilized in conjunction with the Ames Assay (34). A problem with the chromosomal aberration and micronucleus *in vitro* assays is their propensity to produce false positives. There are numerous examples of false positives in the *in vitro* assay when they are evaluated in various *in vivo* assays (46). *In vivo* evaluation of direct mutagenesis detected in the Ames Assay can also be performed, such as the MutaMouse or Big Blue. In reality, a chemical being developed for commercial use that gives a positive result in an Ames Assay will generally not be developed for commercial use unless there is a strong benefit, such as use in cancer chemotherapy.

Assays for immunotoxicity have also been well developed. It should be realized that the use of mouse models, in particular, are not very useful in predicting human immunomodulation effects (50), and as noted above, screening for immunosuppression in animal models is not particularly useful in screening for their carcinogenic activity (42). This is partly due to the fact that mice have a high background of oncogenic retroviruses incorporated in their genome so that production of lymphohematopoietic neoplasms in mice are not particularly relevant to humans (51, 52). The production of the so-called splenic mononuclear cell leukemia (MCL) in rats is likewise primarily found in the F344 rat strain, and it does not appear to be relevant to human cancer risk (53).

In vivo evaluation of immune effects can be performed in rodents, but their relevance to humans is questionable (50). Nevertheless, evaluation in short-term assays for an effect on lymphoid tissues can be performed, including evaluation of hematologic parameters and examination of lymphoid tissues such as thymus, lymph nodes, spleen, mucosa-associated lymphoid tissue (MALT), and bone marrow, which can be performed in animals. However, more useful is a direct evaluation in primary human lymphoid cells which are readily obtainable from human donor samples. These can be utilized for the evaluation of the numerous complex responses of the immune system, including the multiple cell types (B cells, T cells, NK cells, etc.) and for the evaluation of various cytokines. Such an evaluation is already available, albeit requiring considerable resources. Nevertheless, it avoids utilization of animals and is evaluating the response in human cells, not rodent cells.

The difficulty with evaluating effects *in vitro*, whether immune or other effects, involves numerous variables, most of which have not been adequately addressed. To begin with, metabolic activating systems are necessary or the chemical and its potential metabolites have to be evaluated. There are also issues as to whether to utilize immortalized cells or primary cells. Immortalized cells pose considerable difficulty for interpretation since they are already abnormal with numerous genetic abnormalities, commonly including karyotypic abnormalities (54). Utilizing primary cells is preferred, but that raises the question as to which primary cells to use and how many different donors are to be evaluated. Considerations include the age of donors, sex, race, and possibly other variables. How many different primary cell donors need to be evaluated to adequately screen for the human population? This is a significant issue not only for the evaluation of the immune system but for any other parameter in screening. Considerable effort should be made in addressing this issue.

Evaluation of estrogenic activity is already being performed (55) utilizing a variety of *in vitro* systems, including binding and activation of estrogen receptors as well as responses to estrogen activation. If necessary, *in vivo* systems are also available, such as the uterotrophic assay (56). A major consideration in evaluating estrogenic activity is dose.

In the pharmaceutical industry, it has become apparent that there are certain classes of drugs which are known to increase cancer risk because of the receptor target (57). Generally, these involve direct mitogenic effects or cytotoxicity and regenerative effects and are related to the molecular biologic response of the target. Screening for off-site target interactions can be performed by utilizing a variety of *in vitro* receptor-mediated assays. These are generally performed at a dose up to a maximum of 10 µm. This raises the entire issue of what doses are to be used and what limits for any *in vitro* assay. In general, a maximum of 10× or 100× the human exposure level (blood, urine, or other appropriate fluid) should be set. This becomes particularly important when evaluating cytotoxicity since all chemicals can be cytotoxic if a large-enough concentration is utilized in the *in vitro* assays. Certain ranges need to be established as to what is relevant for human exposures. A result in an assay at a high concentration that is positive needs to be put into perspective regarding the human exposure, taking into account thresholds.

The mode of action involving cytotoxicity and regenerative proliferation poses a major challenge for utilizing new methodologies (35, 36). At the present time, this requires *in vivo* screening in animal models, not just in rodents since relevance needs to be evaluated in other larger animal species such as dogs, non-human primates, or mini pigs. Evaluation *in vivo* needs to be made for evidence of increased cell proliferation as well as increased toxicity. This includes evaluation of various markers of cytotoxicity such as hematologic parameters, liver enzymes and kidney markers in blood, and evaluation of various tissues for toxicity and/or increased cell proliferation. Proliferation can be indicated by the presence of hyperplasia in various tissues, but not all. Most notably, some evaluation of DNA replication (labeling index) needs to be made since that is a more sensitive marker for increased proliferation than histopathology is (58, 59). However, not all rodent tissues need to be examined for both cytotoxicity and increased proliferation to screen for human carcinogens. This is because a number of tissues in rodents are not indicative of cancer in humans (Table 1) (2, 60). These include tissues that are present in rodents but not present in humans (forestomach, Harderian gland, and Zymbal's gland) or tumors that occur in animals that do not occur in humans (urinary bladder mesenchymal lesion and rat mononuclear cell leukemia). Moreover, for the most part, endocrine tissues, other than those related to estrogen, are not relevant to human cancer risk, such as the thyroid system, gastrointestinal tract, neuroendocrine tumors, and others. I have also listed tumors such as mouse lung (61), lymphoma (51, 52), and liver (60, 62), and

TABLE 1 Rodent tumors not relevant to humans.

• Rodent organs without human counterpart
■ Zymbal's gland
■ Harderian gland
■ Forestomach
• Rodent tumors without human analog
■ Rat splenic mononuclear cell leukemia
■ Mouse submucosal mesenchymal lesion of bladder (seminal vesicles, uterus)
• Reproductive endocrine tumors
■ Ovary—glanulosa cell
■ Testes—Leydig cell (peritoneal mesothelioma)
■ Endometrium
■ Prostate
• Endocrine organs
■ Thyroid
■ Adrenal cortex
■ Adrenal medulla
■ Pituitary—anterior
■ Pituitary—posterior
■ Parathyroid
■ GI neuroendocrine cells
■ Pancreatic islets
• Questionable relevance
■ Mouse lung
■ Mouse liver
■ Mouse lymphoma
■ Rat pancreas

rat pancreas (63–66) for which considerable evidence supports the conclusion that these are not relevant to human cancer risk. Although evaluations for the potential toxicity of these tissues are important, evaluation in rodents for the prediction of carcinogenesis in humans turns out not to be relevant.

For *in vitro* assays, the major issue is what concentrations are to be evaluated, in addition to the issues of metabolic activation, what cells to be evaluated, and other issues described above. Essentially, all chemicals will be cytotoxic *in vitro* if a high-enough concentration is utilized. This is meaningless. Setting limits on concentrations to be used for *in vitro* assays as described above is essential.

Progress is being made in the development of *in vitro* assays for screening for carcinogens, and this can be utilized in conjunction with shorter-term animal bioassay (1–13 weeks). Combined with the suggested assays described above, this will provide an adequate screen for chemical carcinogenicity with respect to humans (1, 2, 4, 60). A major difficulty in validating new approach methods is as to what basis they are to be compared. Clearly evaluating assays for chemical carcinogenesis based on results in 2-year rodent bioassays is inappropriate since many of the results in the rodent are not relevant to humans. The focus has to be on screening for human carcinogenicity, not rodent carcinogenicity. In addition to the issues raised above with regard to the utilization of *in vitro* assays, there are other considerations. One of these is the reality that there are numerous cellular repair mechanisms available in tissues that protect us from the development of not only carcinogenicity but of toxicity. These have to be evaluated *in vitro* if these *in vitro* systems are to be used for screening for various toxicities, including carcinogenicity. Interactions of multiple cell types in a tissue are also critical and need to be evaluated. To some extent, these issues are being addressed utilizing 3D cultures, but these need considerable additional development before they can be utilized more broadly. They certainly can be used already to address specific biologic questions.

Conclusions

Most scientists now agree that the long-term bioassay in rodents is no longer appropriate for screening for human carcinogenicity risk. Many agencies have already stopped performing such studies, such as the US National Toxicology Program. Various regulatory agencies are moving toward abandoning the requirement for a 2-year bioassay, but additional effort is necessary. Most importantly, changes in approach by regulatory agencies will require changes in guidelines, and even laws, for certain types of chemicals such as pharmaceuticals, agrochemicals, cosmetics, food ingredients, and other types of chemicals.

In this paper, I have presented a proposed approach for screening for chemical carcinogens based on mode of action, with a realization that only four basic modes of action are relevant to human carcinogens: DNA reactivity and mutagenesis, immunomodulation, increased estrogenic activity, and cytotoxicity with consequent regeneration. All but DNA reactivity are based on evaluations related to increased cell proliferation. Evaluation for these affects can be performed using alternative assays, including *in vitro*, *in silico*, and *in vivo*. Some basic questions regarding *in vitro* remain, such as metabolic activation, cell types to be evaluated, and concentrations to be utilized. Extrapolation to the *in vivo* situation is essential, with increasing progress being made on *in vitro* to *in vivo* extrapolation being accomplished by utilizing physiologically based pharmacokinetic (PPBK) models. The proposed approach for screening for carcinogenicity will be less costly, take less time, require fewer animals, and be more relevant to human risk than the use of the two-year bioassay in rodents. Of course, if an assay shows a positive signal, more extensive dose response analyses will be required to evaluate the actual risk at human exposures.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee (IACUC). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SC: Writing – review & editing, Writing – original draft.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Investigating phenotypic plasticity due to toxicants with exposure disparities in primary human breast cells *in vitro*

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Introduction: Breast cancer is the second most diagnosed cancer, as well as the primary cause of cancer death in women worldwide. Of the different breast cancer subtypes, triple-negative breast cancer (TNBC) is particularly aggressive and is associated with poor prognosis. Black women are two to three times more likely to be diagnosed with TNBCs than white women. Recent experimental evidence suggests that basal-like TNBCs may derive from luminal cells which acquire basal characteristics through phenotypic plasticity, a newly recognized hallmark of cancer. Whether chemical exposures can promote phenotypic plasticity in breast cells is poorly understood.

Methods: To investigate further, we developed a high-content immunocytochemistry assay using normal human breast cells to test whether chemical exposures can impact luminal/basal plasticity by unbiased quantification of keratin 14 (KRT14), a basal-myoepithelial marker; keratin 8 (KRT8), a luminal-epithelial marker; and Hoechst 33342, a DNA marker. Six cell lines established from healthy tissue from donors to the Susan G. Komen Normal Tissue Bank were exposed for 48 hours to three different concentrations (0.1μM, 1μM, and 10μM) of eight ubiquitous chemicals (arsenic, BPA, BPS, cadmium, copper, DDE, lead, and PFNA), with documented exposure disparities in US Black women, in triplicate. Automated fluorescence image quantification was performed using Cell Profiler software, and a random-forest classifier was trained to classify individual cells as KRT8 positive, KRT14 positive, or hybrid (both KRT8 and KRT14 positive) using Cell Profiler Analyst.

Results and discussion: Results demonstrated significant concentration-dependent increases in hybrid populations in response to BPA, BPS, DDE, and PFNA. The increase in hybrid populations expressing both KRT14 and KRT8 is indicative of a phenotypically plastic progenitor-like population in line with known theories of carcinogenesis. Furthermore, BPA, BPS, DDE, and copper

produced significant increases in cell proliferation, which could be indicative of a more malignant phenotype. These results further elucidate the relationship between chemical exposure and breast phenotypic plasticity and highlight potential environmental factors that may impact TNBC risk.

KEYWORDS

breast cancer, triple negative breast cancer, phenotypic placticity, immunocytochemistry, toxicology, environment, disparities

1 Introduction

Breast cancer alone accounts for 31% of all new cancer diagnoses in women, and incidence rates have been increasing by approximately 0.5% each year (1). Breast cancer is also responsible for 15% of all female cancer-related deaths each year; however, there are stark contrasts in outcomes and survival across races and ethnicities (1). Despite a 4% lower incidence rate compared to non-Hispanic White women, non-Hispanic Black women have a 40% higher breast cancer-associated mortality rate compared to non-Hispanic White women (1). Furthermore, relative to non-Hispanic White women, non-Hispanic Black women have a two to three times higher risk of developing triple-negative breast cancer (TNBC), an extremely aggressive and heterogenous subtype of breast cancer with no targeted therapy (2).

There is no current data at a molecular or biological level that can fully explain the etiology of these disparities (3). Environmental factors, such as chemical exposure disparities, may play a role in the disparate incidence of TNBC in African American women. However, these drivers are poorly understood. Compared to other demographics, African American women, on average, are exposed to elevated levels of multiple toxicants, including lead, cadmium, arsenic, p,p′-dichlorodiphenyldichloroethylene (DDE), bisphenol S (BPS), and perfluorononanoic acid (PFNA) (4–6). Additionally, bisphenol A (BPA) levels are higher in lower-income individuals, and African American women are more likely to face socioeconomic adversity (7, 8). Many of these disparate exposures can be directly linked to unequal living conditions, associated with historical systemically racist practices, such as redlining, that do not provide an adequate amount of safety and protection against environmental exposures (9). Dietze et. al (3) proposed that neighborhood level factors may be the intersection between disparities and the aggressive nature of TNBC in African American women. Independent of socioeconomic factors, unjust beauty norms result in further disparate exposures from harmful chemicals in targeted personal care products (10). Hair texture preference and colorism have led to widescale production of hair straightener and skin lightening beauty products that are often unregulated and filled with deleterious chemicals and contaminants (10). Chemical hair straightening products, in particular, are disproportionately purchased by African American women and have been associated with premature breast development and increased risk of premenopausal breast cancer (10). While the mechanisms implicating disparate chemical exposures as drivers of aggressive breast cancer are poorly understood, it is becoming increasingly apparent that chemical exposures may impact breast cancer risk (11, 12).

A process that contributes to breast cancer progression is phenotypic plasticity, a newly defined Hallmark of Cancer (13, 14). Phenotypic plasticity describes a cell's ability to transition and acquire another cellular constitution in response to environmental stress (15). The result is a cell that exhibits "hybrid" characteristics, simultaneous expression of phenotypic characteristics of two or more cell types. These hybrid cells can be present in normal mammary tissue; however, they are primarily observed in malignant cells, and have been shown to promote tumorigenesis and metastasis of breast cancer (15, 16). Of TNBC cases, 80.6% are considered basal-like breast cancers (17). Basal-like breast cancers express genes consistent with normal myoepithelial cells such as KRT14 (17). Numerous other genes can be useful as markers for other cell types, such as KRT8, a known luminal marker gene (18). Overlap of KRT14 and KRT8 expression is indicative of hybrid populations (15), and single-cell analyses have revealed that these hybrid "basoluminal" populations increase with age in the normal mammary gland (19). KRT8 and KRT14 are both intermediate filament proteins that not only reflect the epithelial cell type but can also be used to indicate growth and differentiation factors (20). Upregulation of KRT14 has been associated with a more invasive breast cancer phenotype, while increased abundance of KRT8 is common in malignant cells (21, 22).

For estrogen receptor-negative breast cancers, such as TNBC, there is mounting evidence to suggest that chemical exposures may increase risk for breast cancer by inducing phenotypic plasticity. Recent studies have implicated cadmium, arsenic, and BPA as environmental chemicals capable of inducing phenotypic plasticity in normal human breast cells (1–3, 5–7, 9, 11, 12, 14–16, 18, 19, 22–49). The goal of the present study was to further characterize the association between exposure to chemicals with documented exposure disparities and phenotypic plasticity in primary normal breast cells from diverse donors. Based on previous work that highlights known chemical exposure disparities, as well as prior evidence of chemically induced phenotypic plasticity, we hypothesized that chemicals with known

exposure disparities in African American women would promote phenotypic plasticity and hybrid states in normal breast epithelial cells. To test this hypothesis, we optimized a novel high-throughput imaging assay using luminal and basal markers in normal human breast cells. This assay was employed to examine phenotypic plasticity in response to chemical dosing in primary breast cells and further elucidate the environmental factors and disparities present in breast cancer.

2 Methods

We developed a high-throughput immunocytochemistry assay to quantify KRT8 and KRT14 staining of toxicant-treated and negative control/vehicle-treated primary human cell lines. The toxicants used were lead acetate (Sigma-Aldrich 316512), copper chloride (Sigma-Aldrich 222011), cadmium chloride (Sigma-Aldrich 202908), sodium arsenite (Sigma-Aldrich S4700), p,p′-DDE (Chem Service N-10875), BPA (Sigma-Aldrich 239658), BPS (Sigma-Aldrich 103039), and PFNA (Sigma-Aldrich 394459). Chemicals were chosen based on a previous study that identified known exposure disparities between non-Hispanic Black women and non-Hispanic White women (5). A diverse set of normal human breast cell lines grown from normal breast punch biopsy tissues obtained from the Susan G. Komen Tissue Bank were used to test these exposures and observe differences in response to chemical dosing *in vitro*. The samples were all from nulliparous women who either self-identified as African American or European American (three cell lines for each group) and matched for age, BMI, and date of last menstrual period (Supplementary Table 1).

Following optimization of our novel assay, we cultured each cell line and treated cells with human-relevant concentrations of our chosen chemicals. Following an incubation period, we employed our immunostaining protocol and imaged plates on a high-content imaging microscope. To quantify and analyze dosing effects, as well as interindividual differences, we created Cell Profiler Analyst pipelines. Quantitative analysis and data visualization were completed in R Studio software using the results obtained from Cell Profiler.

2.1 Cell culture

Cell lines (Supplementary Table 1) were established from tissue by enzymatic and mechanical digestion as previously described (15, 50). The resulting cryopreserved cell lines were thawed and cultured in accordance with previously established methods (15, 38). Primary cells grew to confluence in T-75 flasks with irradiated mouse J2 fibroblasts, which provide an optimal growth environment (15).

Once confluent, cells were diluted to 50,000 live cells per ml in F-media, to be plated at 1,500 cells (30 µl) per well in collagen-coated 384-well plates (Corning Biocoat Collagen I-rat tail collagen type I Product Number: 354667). Cells were plated 24 h prior to dosing and placed in a humidified incubator at 37°C/5% CO2 overnight.

F-media was prepared by combining 500 ml of DMEM (Fisher, cat. no. 11965092), 50 ml of heat-inactivated fetal bovine serum (Sigma Aldrich, cat. no. F4135), 5.5 ml of 200 mM L-glutamine (Gibco, cat. no. 25–030-081), 5.5 ml of 100X Pen-Strep (Fisher, cat. no. 15140122), 187 ml of F-12 (Fisher, cat. no. 11765054), 194.48 µl of 96 µg/ml of hydrocortisone (Stem Cell, cat. no. 07925), 935 µl of 4 mg/ml of insulin (Fisher, cat. no. 12585014), 8.98 µl of 10 µg/ml of EGF (Stem Cell, cat. no. 78006.1), 62.83 µl of 1.2 µM cholera toxin (Sigma Aldrich, cat. no. C8052), and 623.83 µl of 12 mM Y-27632 inhibitor (Stem Cell cat. no. 72302).

2.2 Chemical dosing

Chemical concentrations were prepared using serial dilutions, and each chemical was diluted to three concentrations: 100 nM, 1 µM, and 10 µM. These concentrations were chosen given established biological relevance from previous work from our group that established benchmark concentrations *in vitro* linked to biomarker concentrations from human population data from the National Health and Nutrition Examination Survey (6, 22). Sala-Hamrick et al. established median benchmark concentrations for each of the toxicants used in this study using RNA sequencing and found large impacts between 10 nM and 10 µM (22). Water (Invitrogen 10–977-015) and DMSO (Sigma-Aldrich D2650) served as the vehicles for heavy metals and organics, respectively. Three replicates were assessed per concentration for each chemical on each cell line. Following dosing, well plates were placed back in the humidified incubator at 37°C/5% CO_2 for 48 h prior to immunostaining.

2.3 Immunostaining and imaging

After exposure, cells were stained for expression of KRT8 and KRT14, along with the nuclear stain Hoechst 33342. The following reagent concentrations have been optimized and were used for each cell line: 4% paraformaldehyde was prepared by diluting 925 µl of 16% paraformaldehyde (Thermo Fisher Scientific cat. no. AA433689M) in 3,150 µl of PBS (Gibco cat. no. 10–010-049). PBST was prepared by adding 50 µl of 100% Tween20 (Thermo Fisher Scientific cat. no. BP337) to 49.95 ml of PBS. Triton X (0.1%) was prepared by diluting 3.7 µl of 100% Triton X (Sigma-Aldrich cat. no. T8787) in 3.7 ml of PBST. Blocking buffer was prepared by dissolving 83.33 mg of glycine (Thermo Fisher Scientific cat. no. AAA1381636) in 3.7 ml of PBST and 493.3 µl of 7.5% BSA (Gibco cat. no. 50–121-5315). BSA (1%) was prepared by adding 493.3 µl of BSA to 3.7 ml of PBST. The antibody solution was prepared by adding 24.6 µl of Anti-Cytokeratin 8 (1:150 ratio) (Alexa Fluor 488, Clone number EP 1628Y; Isotype IgG) and 37 µl of Anti-Cytokeratin 14 (1:100 ratio) (Alexa Fluor 647, Clone number EP 1612Y; Isotype IgG) to 3.7 ml of 1% BSA in PBST. The counterstaining solution was prepared by adding 1.9 µl of Hoechst 33342 (Thermo Fisher Scientific cat. no. H3570) and 3.7 ml of 1% BSA in PBST.

After 48 h of incubation (37°C), dosed cells were washed with PBS and then fixed with 4% paraformaldehyde in PBS (pH 7.4). 40 µl of 4% paraformaldehyde was dispensed into each well, and the plate was centrifuged once at 500*g* for 30 s. Following centrifugation, plates were allowed to sit at room temperature for 10 min. The 4% paraformaldehyde was then aspirated, and cells were washed with PBS again prior to permeabilization. Cells were permeabilized with 0.1% Triton-X 100 in PBS. Triton-X 100 (0.1%) was also dispensed at 40 µl per well, and the plate was centrifuged again at 500*g* for 30 s. The plate then sat at room temperature for 10 min before being washed twice with PBS. To prevent any non-specific staining, cells were blocked using a 1% BSA and glycine in PBST blocking buffer. 40 µl of blocking buffer was added to each well, the plate was centrifuged at 500*g* for 30 s, and then sat at room temperature for 1 h. The cells were not washed after blocking; the buffer was aspirated, and 40 µl per well of the antibody solution was added. The plate was centrifuged once more at 500*g* for 30 s and then covered and allowed to incubate overnight (8–12 h) in a 4°C refrigerator. The next day, the antibody solution was decanted, and cells were washed with PBST three times in the dark prior to counterstaining. 40 µl of the counterstaining solution was added to each well, and the plate was centrifuged at 500*g* for 30 s. The plate was then covered and allowed to rest at room temperature for 1 h in the dark. Cells were then washed three times with PBST before imaging with the Yokogawa Cell Voyager 8000 (CV8000) microscope. Automated plate imaging was performed on the CV8000 with a ×20/1.0NA water immersion objective lens, 50-µm pinhole, with three channels; 405 nm (Hoescht), 488 nm (KRT8), and 647 nm (KRT14). Laser power for each channel was adjusted to ensure optimal signal-to-noise ratios, laser-based autofocus was performed at each field, and nine fields per well were imaged.

2.4 Fluorescence microscopy analysis

Images obtained from the CV8000 were analyzed using Cell Profiler and Cell Profiler Analyst software (45, 46). To analyze the acquired images, we optimized a quality control (QC) pipeline in Cell Profiler, as well as an analysis pipeline in Cell Profiler. Images ran through the QC pipeline first, and a gentle boosting classifier was created to identify images that were oversaturated for each cell line in Cell Profiler Analyst to create unique flag rules. These rules were then inputted into the Cell Profiler Analysis pipeline, and oversaturated images were removed and excluded from further analysis. In the Cell Profiler Analysis pipeline, nuclei were set as primary objects, while an overlay of both keratins was used to identify individual cells as secondary objects. Mean intensity of KRT8 and KRT14 were measured on a per-cell basis to ensure population classification was accurate in the Cell Profiler classification. Total cell counts were quantified using the primary object, including any cells that did not express either keratin protein.

Following the completion of the analysis pipeline, we trained a random forest classifier for each cell line in Cell Profiler Analyst to recognize and identify the different antibody staining patterns, as well as any remaining J2 fibroblasts, which were identified by Hoechst and their unique nuclear staining. Classifiers were trained to at least 90% accuracy. These classifiers were specifically grouped into four categories: luminal (KRT8+/KRT14−), myoepithelial (KRT14+/KRT8−), hybrids (KRT8+/KRT14+), and cells that did not express either protein (KRT8−/KRT14−) (Supplementary Figure 1).

Output from classifications were saved as CSV documents that scored each image and identified the number of each phenotype across all treatments and cell lines. This CSV text document was then inputted into R software (1.4.1717) for further analysis and data visualization.

2.5 Data analysis

To perform statistical analysis, CSV documents generated from Cell Profiler Analyst were uploaded and used to compare the proportion of cells that fluoresced in each category (KRT8, KRT14, and hybrids) by treatment and concentration. J2 fibroblasts were excluded from further analysis, although primary cells that were negative for either KRT8 or KRT14 were still accounted for in the total cell counts. To ensure the accuracy of the Cell Profiler Analyst classifier, validation of the classified cells (KRT8, KRT14, and hybrid) were verified by comparing KRT8 and KRT14 staining intensity data (Figure 1 shows an example of this

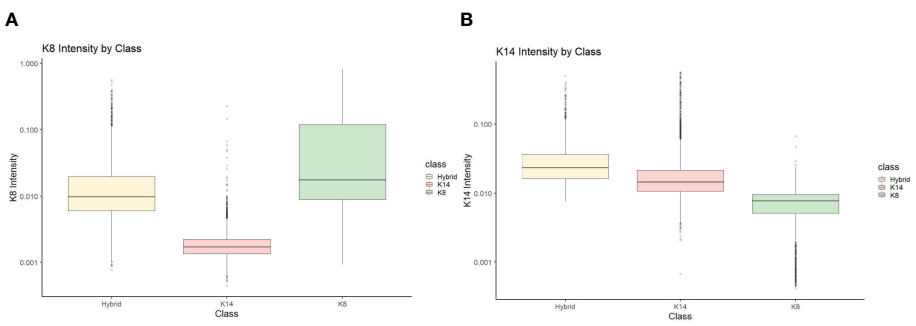

FIGURE 1
KCR 8195 classification intensity validation for **(A)** KRT8 intensity and **(B)** KRT14 intensity.

for one line, KCR8195. Supplementary Figures 2–6 show the validation data from the remaining five lines.).

Wilcoxon signed rank-sum tests were utilized to quantify differences in the proportions of KRT8, KRT14, and hybrids by treatment and concentration for a given cell line, compared to the appropriate vehicle control. Significance was measured using Wilcoxon signed rank-sum tests ($p < 0.05$), and three criteria were used to indicate phenotypic plasticity: 1. Significant increases in hybrid populations, and/or 2. Significant and complementary shifts between hybrid populations and KRT8 or KRT14 populations, and/or 3. Significant and complementary shifts between KRT8 and KRT14 populations. Conditions that met at least one of the three criteria were indicators of phenotypic plasticity in this study. Cell count was also compared by treatment and concentration by conducting Wilcoxon signed rank-sum tests. Significant decreases in hybrid populations without a concomitant increase in another cell population were not counted as phenotypic plasticity; while some phenotypic plasticity may be present, changes in total cell count after dosing could have also contributed to population decreases.

3 Results

3.1 Concentration-response effect on cell populations with fluorescence microscopy

Images obtained from the CV8000 were analyzed for nuclear (Hoescht), luminal (KRT8), myoepithelial (KRT14), and hybrid (KRT8/KRT14 overlap) expression (Figure 2).

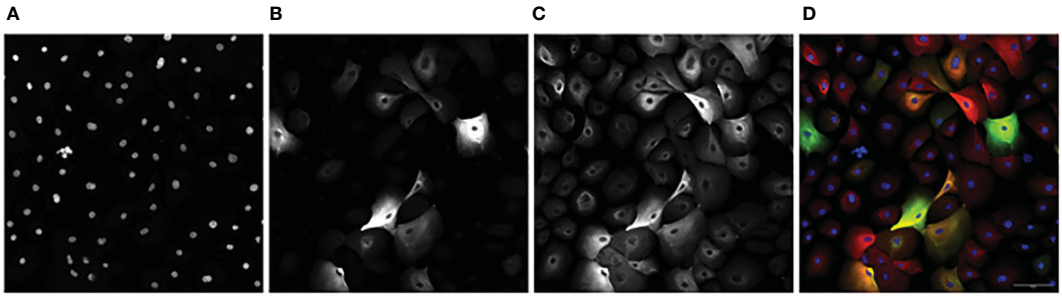

FIGURE 2
An example image series of KCR 8195 dosed with 1 µM of PFNA: **(A)** Nuclei. **(B)** KRT8. **(C)** KRT14 **(D)** Composite with KRT14 immunofluorescence shown in red, KRT8 immunofluorescence shown in green, with Hoechst shown in blue. Orange and yellow cells represent KRT8/KRT14 basoluminal hybrids. Scale bar represents 100 µm.

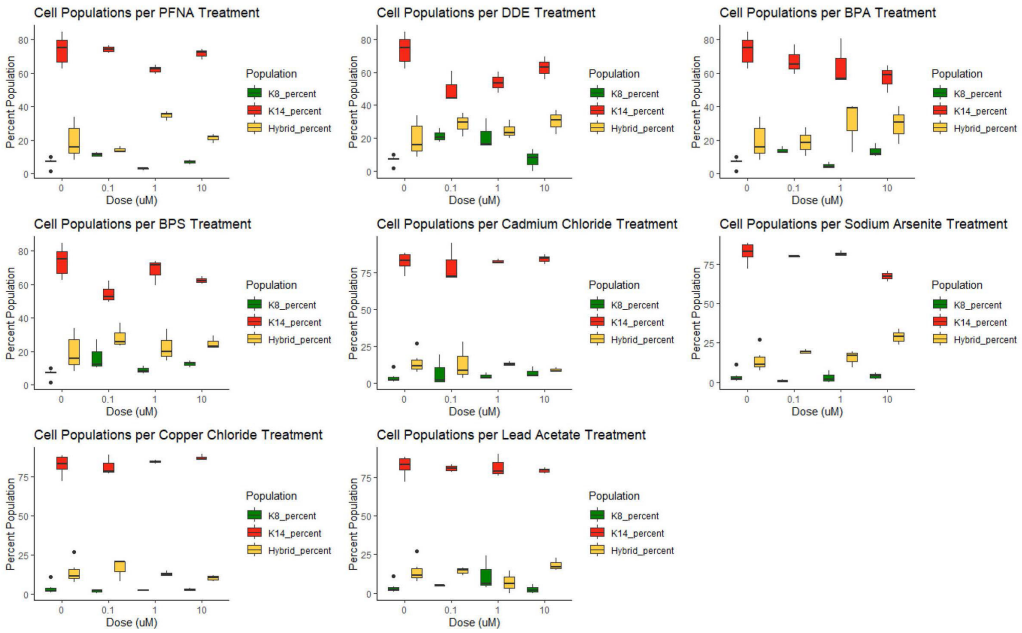

FIGURE 3
KCR 8195 combined populations (%) for each chemical compared to each associated control (0 µM concentration). Significance determined by Wilcoxon signed rank-sum tests and reported in Figures 4, 5 and Supplementary Figures 12–15.

KCR7889, KCR7953, KCR8195, and KCR8580 primarily expressed myoepithelial cells in both the dosed and control populations (Figure 3 and Supplementary Figures 8, 9 and Supplementary Figure 11). KCR7518 and KCR8519 primarily expressed luminal cells in both the dosed and control populations (Supplementary Figures 7, 10). Basal populations of hybrid cells were present in each cell line as well. To identify the effect of chemical concentrations on phenotypic plasticity, we used machine learning to quantify the amount of each cell type under each dosed condition and compared these values to controls (Figure 3 and Supplementary Figures 7–11). KCR 8195, in particular, had a high cell count as well as a large presence of hybrid cells at baseline and was subsequently chosen for presentation. As shown in Figure 3, KCR 8195 had concentration-dependent decreases in KRT14-marked cells and subsequent increases in KRT8 and hybrid cell populations, with significant shifts seen in low concentrations of p,p′-DDE and BPS.

3.2 Significant changes in cell type as a marker for phenotypic plasticity

Significant changes in cell type, either from luminal to myoepithelial, myoepithelial to luminal, or increases and decreases of hybrid populations were used as markers of phenotypic plasticity. Significance was determined using Wilcoxon signed rank-sum tests ($p < 0.05$). In controls, four of the six cell lines were predominately myoepithelial; however, each displayed significant changes in the KRT8 luminal marker under dosed conditions (Supplementary Figures 12, 13). KCR 7889 demonstrated the most myoepithelial to luminal plasticity, with significance found in 10 different concentrations across each of the organic chemicals that were used (Supplementary Figure 12). KRT 8580 demonstrated the most phenotypic plasticity, with significant myoepithelial to hybrid shifts as well as luminal to hybrid shifts in eight different concentrations across each of the organic chemicals that were used (Supplementary Figures 12, 13). The two cell lines that presented predominately luminal markers basally also demonstrated significant plasticity. Notably, KCR 7518 had a significant shift between hybrid to KRT8 luminal populations in one concentration of BPS, and KCR 8519 had a significant increase in KRT14 myoepithelial cells following exposure to two concentrations of lead acetate (Supplementary Figures 12, 15). Overall, most cell lines demonstrated the potential for phenotypic plasticity; however, KCR 8580 had the highest proportion of hybrid cells at baseline and demonstrated the most significant transitions between luminal and myoepithelial cell types (Figures 4, 5 and Supplementary Figures 12–15).

3.3 Changes in cell count as an implication for requisite future work

The average cell counts of each cell line varied; however, water control wells and wells that were dosed with metals diluted in water were more robust to cell number changes than DMSO control wells

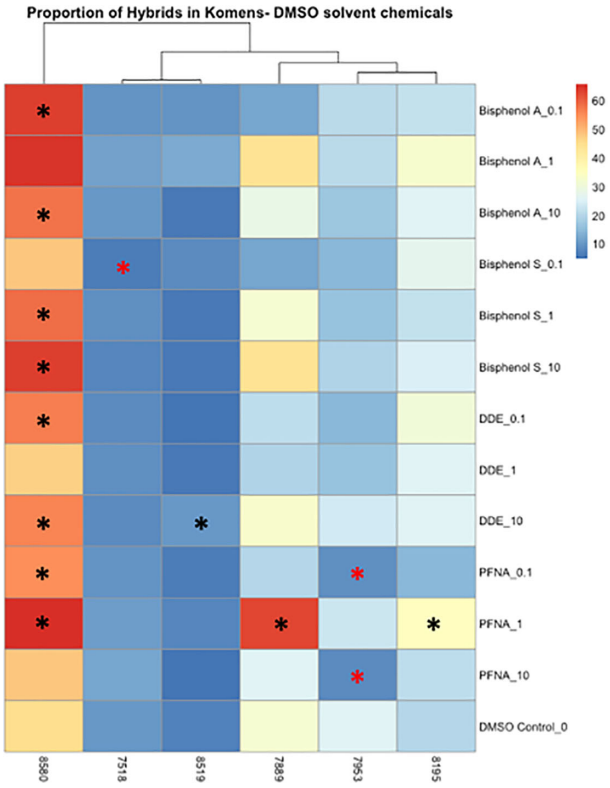

FIGURE 4
Heatmap depicting the percent of the cells in each treatment condition, which are in a hybrid state, for organic chemical-treated cells only. Differences in hybrid percentages between a given treatment and the DMSO control were determined by Wilcoxon signed rank-sum tests and denoted by an * ($p < 0.05$). Increases in hybrid populations are represented by a black asterisk, while decreases in hybrid populations are represented by red asterisks.

or wells that were dosed with chemicals diluted in DMSO across cell lines (Figure 6). Significant decreases in cell number occurred in multiple chemicals at multiple concentrations. KCR 8195 had significant decreases in cell number in 100 nm and 10 µm of BPA, 100 nm and 1 µm of BPS, 1 µm of cadmium, 1 µm of arsenic, and across all lead concentrations (Supplementary Figure 16). KCR 7518 had significant decreases in cell number throughout all concentrations of arsenic (Supplementary Figure 17). KCR 7889 had significant decreases in cell number in 100 nm of cadmium, 10 µm of arsenic, and 10 µm of lead (Supplementary Figure 18). KCR 7953 only saw a significant decrease in cell number in 10 µm of arsenic (Supplementary Figure 19). KCR 8519 had significant decreases in cell number in 10 µm of cadmium, 1 and 10 µm of arsenic, and 10 µm of copper (Supplementary Figure 20). KCR 8580 only saw a significant decrease in cell number in 10 µm of arsenic (Supplementary Figure 21).

4 Discussion

There are consistent data that implicates chemical exposures as drivers of breast cancer. Cadmium, arsenic, and BPA have each been associated with epigenetic modifications in normal human

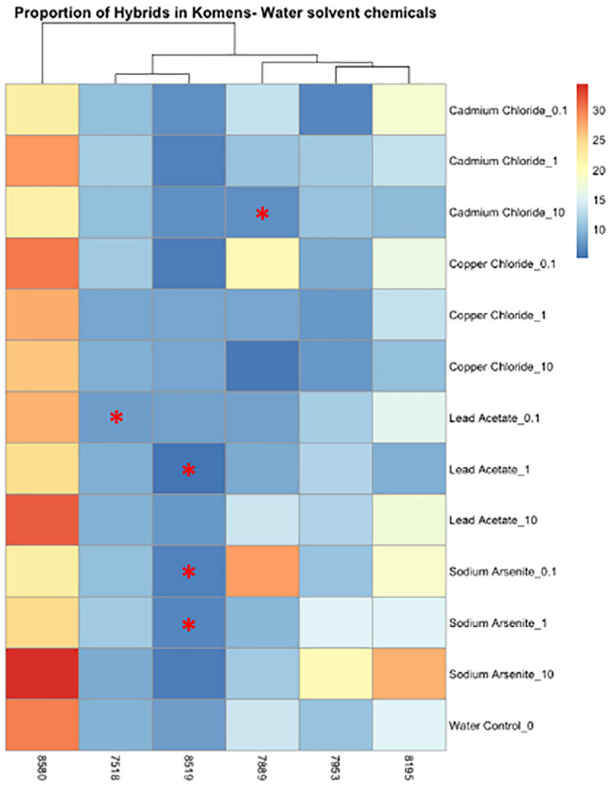

FIGURE 5
Heatmap depicting the percent of the cells in each treatment condition, which are in a hybrid state, for metal-treated cells only. Differences in hybrid percentages between a given treatment and the water control were determined by Wilcoxon signed rank-sum tests and denoted by a * ($p < 0.05$). Increases in hybrid populations are represented by a black asterisk, while decreases in hybrid populations are represented by red asterisks.

breast cells; previous work has demonstrated that each of these chemicals may be drivers of malignant phenotypic plasticity in normal human breast cells via basoluminal transitions (23, 24, 49). In addition to these three chemicals, prior research has highlighted that exposure disparities of lead, p,p′-DDE, PFNA, BPS, and copper exist for African American women (6). In this study, we sought to further characterize the potential for disparate chemical exposures to induce malignant phenotypic plasticity *in vitro*.

Phenotypic plasticity was identified by significant increases in hybrid populations, or through significant and complementary changes between cytokeratin 8 and cytokeratin 14 populations, a summary of which is shown in Table 1. Collectively, these findings suggest that arsenic, BPA, BPS, DDE, and PFNA are capable of stimulating basoluminal transitions in normal human breast cells. This is consistent with previous findings that identified low *in vivo* and *in vitro* concentrations, between 7 nM and 2 µM of BPA, 1 nM and 3 µM of BPS, 20 nM to 0.75 µM of DDE, and 1 nM to 1.65 µM of PFNA, could induce transcriptomic changes in breast cells (22).

Changes in total cell count in response to exposures are also highlighted in Table 1. Collectively, these results may demonstrate the cytotoxicity of arsenic, cadmium, and lead at these concentrations, which are consistent with previous findings (23, 24, 26, 43). Sodium arsenite, in particular, has been shown to induce apoptosis in MCF-7 breast cancer cells at concentrations greater than 5 µM (43). The interindividual response to metal exposure, as reflected by cell numbers being impacted in cultures from some individuals but not others, is also worthy of exploration in future studies to understand the factors that promote or prevent toxic effects from a given substance.

Results shown in Table 1 may also demonstrate the proliferative effects of BPA, BPS, and DDE, which are consistent with previous findings (22, 30, 42). These proliferative effects have been attributed to genetic and epigenetic modifications following exposures in non-tumorigenic MCF-10A breast epithelial cells, with specific upregulation of human epidermal growth factor following BPS exposure, and upregulation of the PCNA gene following DDE exposure (22, 30, 42). We also recently found that low-concentration DDE (25 nM) activates Wnt signaling in MCF-10A cells, as reflected by increased translocation of beta catenin to the nucleus, which may, in part, explain increased cellular proliferation (47). The mechanisms behind the proliferative effects associated with these chemicals in these normal primary lines from diverse donors is worthy of future exploration.

Interindividual differences were present between cell lines at baseline; some lines were predominately luminal, while others were predominately myoepithelial. Among the cell lines used in this study, those that were predominately myoepithelial or contained more hybrid cells at a baseline level demonstrated increased plasticity compared to the cell lines that contained predominately luminal cells in the control. Significant phenotypic plasticity markers were analyzed for each individual, the three European American cell lines never (KCR 7953) or rarely (one for KCR7518 and three for KCR 7889) had significant plasticity, while the African American cell lines more commonly demonstrated plasticity (two for KCR 8519, three for KCR 8195, and eight for KCR 8580). Although the low sample size dictates that no conclusive data can be deduced regarding TNBC disparities, this represents an approximate 3.25-fold increase in phenotypic plasticity markers among the African American cell lines compared to the European American cell lines. Future work is requisite to validate these findings, to test additional diverse cell lines to further elucidate these trends, and to examine the impact of exposures of longer duration, which more accurately model the chronic nature of many of the exposures under investigation. Additional work should also examine the impacts of these toxicants *in vivo* and in well-characterized human tissue samples, as phenotypic plasticity in culture may not reflect what is possible when cells are constrained in a tissue microenvironment. Future research should also consider the larger exposome of toxicants that each individual may be subjected to, as previous work has identified that mixtures of chemicals may increase the aggression of breast cancer cells and promote additional Hallmarks of Cancer, such as invasion and metastasis (51). As we know that people are exposed to complex mixtures of toxicants, reconstructing chemical mixtures at human relevant concentrations, particularly in the context of exposure disparities, and assessing the effects of the mixtures in primary cell lines would be an exciting and important next step.

Additional work is also necessary to further characterize the mechanisms underlying both the cytotoxicity and cellular proliferation

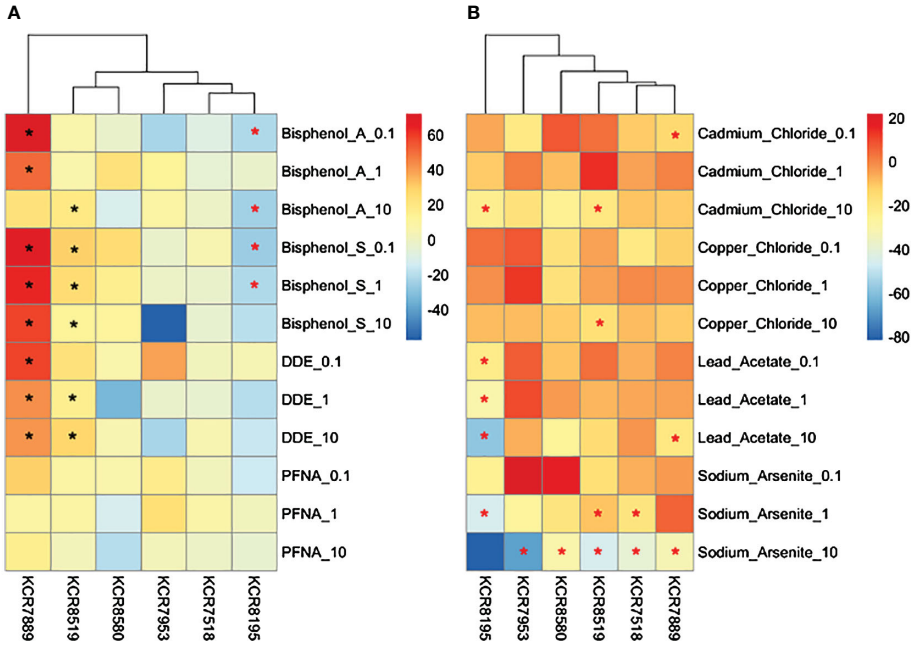

FIGURE 6
Heatmaps depicting changes in cell counts (% relative to control) per sample for **(A)** DMSO solvent chemicals and **(B)** water solvent chemicals. Chemical concentrations are measured in μM—0.1 is equal to 100 nM. Differences in cell counts relative to control were determined by Wilcoxon signed rank-sum tests and denoted by a * ($p < 0.05$). Increases in cell counts are represented by a black asterisk, while decreases are represented by red asterisks.

TABLE 1 Summary of phenotypic plasticity for each cell line as indicated by increases in hybrid populations and/or shifts between KRT 8 and KRT 14 populations, as well as significant total cell count increases or decreases by cell line.

Cell Line	Increase in Hybrids	KRT 8/KRT 14 Shift	Increase in Cell Count	Decrease in Cell Count
KCR 7518	None	Decrease in hybrid populations seen with subsequent increase in KRT 8 populations in 100 nM BPS	None	Decrease in cell count in all concentrations of Arsenic
KCR 7889	Increase in hybrid populations in 1 μM PFNA and 100 nM Arsenic	Decrease in KRT 14 populations seen with subsequent increase in KRT 8 populations in 10 μM BPS	Increase in cell count in 1 μM and 10 μM concentrations of BPA, and across all concentrations of DDE and BPS	Decrease in cell count in 100 nM of Cadmium, 10 μM of Arsenic, and 10 μM of Lead
KCR 7953	None	None	None	Decrease in cell count in 10 μM Arsenic
KCR 8195	Increase in hybrid populations in 1 μM PFNA and 100 nM BPS	Decrease in KRT 14 seen with subsequent increases in KRT 8 populations in 100 nM DDE	None	Decrease in cell count in 100 nM and 10 μM BPA, 100 nM and 1 μM BPS, 1 μM Cadmium, 1 μM Arsenic, and across all Lead concentrations
KCR 8519	Increase in hybrid populations in 10 μM DDE	Decrease in KRT 14 populations seen with subsequent increase in KRT 8 populations in 100 nM BPA	Increase in cell count in 1 μM and 10 μM DDE, 10 μM BPA, and across all concentrations of BPS	Decrease in cell count in 10 μM Cadmium, 1 μM and 10 μM Arsenic, and 10 μM Copper
KCR 8580	Increase in hybrid populations in 100 nM and 10 μM PFNA, 100 nM and 10 μM DDE, 1 μM and 10 μM BPS, and 100 nM and 10 μM BPA	None	None	Decrease in cell count in 10 μM Arsenic

associations found in some of these chemicals as well—this work is currently underway. Additional cytotoxicity assays, in particular, may be useful in elucidating the mechanisms behind the alterations in cell number, and the interindividual variation in these alterations, due to increasing concentrations of arsenic, lead, and cadmium. This work also sets the stage for a mechanistic interrogation of how environmental factors can promote cellular plasticity and cell state transitions, for example, by interrogating epigenetic changes (27). Understanding the biological drivers of luminal–basal phenotypic plasticity, and how toxicants can perturb these processes, would provide substantial insights into how chemicals may impact the risk of aggressive breast cancers. Luminal–basal hybrid cells have been identified in normal breast tissue using single-cell profiling (15, 19, 50). Intriguingly, the proportion of these hybrid cells increases with age suggesting aging-associated alterations—perhaps the accrual of a lifetime of exposure to environmental stressors—can promote the emergence or expansion of these cell populations (19). In cancer, experimental studies suggest that basal breast cancers derive from luminal cell populations (33). Studies of estrogen receptor-positive breast cancer MCF7 cells exposed to arsenic long term *in vitro* highlight suppression of epithelial markers, induction of basal markers, and reduced expression of hormone receptors potentially linked to alterations in signaling of stem cell-associated pathways like Hedgehog (24). Research exploring how exposure to additional chemicals and a broader range of concentrations may drive similar effects in primary cells is now requisite. While concentrations chosen for this study were chosen based on previous benchmark concentration modeling based on RNA-seq data, a broader concentration range could be tested in the future, particularly to understand and quantify low concentration effects and potential non-monotonic concentration responses (22). Assaying additional markers may also prove useful in elucidating the role environmental factors may play in the progression of cancer. For example, expression of CD44, CD24, and ALDH1A3 can quantify epithelial and mesenchymal stem cell states in breast cancer and normal breast tissue (37, 50). Associations drawn from these experiments would further solidify the role environmental factors play in TNBC development linked to dysregulated stemness. Further research focused on the specific mechanisms, including epigenetic changes, by which environmental exposures can promote cellular plasticity will provide key insights into how chemical exposures may promote aggressive breast cancers. Overall, these data support that phenotypic plasticity can be modulated by toxicants with disparate exposures *in vitro* in primary human breast cells providing supporting evidence that exposure to these toxicants may be able to perturb this newly defined Hallmark of Cancer.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

JS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. KP: Formal analysis, Methodology, Validation, Writing – review & editing. AT: Conceptualization, Formal analysis, Visualization, Writing – review & editing. LS: Writing – review & editing. JZS: Formal analysis, Resources, Writing – review & editing. JC: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2024.1411295/full#supplementary-material

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A case for the study of native extracellular vesicles

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Three main areas of research revolve around extracellular vesicles (EVs): their use as early detection diagnostics for cancer prevention, engineering of EVs or other enveloped viral-like particles for therapeutic purposes and to understand how EVs impact biological processes. When investigating the biology of EVs, it is important to consider strategies able to track and alter EVs directly *in vivo*, as they are released by donor cells. This can be achieved by suitable engineering of EV donor cells, either before implantation or directly *in vivo*. Here, we make a case for the study of native EVs, that is, EVs released by cells living within a tissue. Novel genetic approaches to detect intercellular communications mediated by native EVs and profile recipient cells are discussed. The use of Rab35 dominant negative mutant is proposed for functional *in vivo* studies on the roles of native EVs. Ultimately, investigations on native EVs will tremendously advance our understanding of EV biology and open novel opportunities for therapy and prevention.

KEYWORDS

cancer, intercellular communication, B cells, extracellular vesicles, *in vivo*

Introduction

A key mode of communication between cells involves extracellular vesicles (EVs), which incorporate donor cell-derived signals (both membrane-bound and intracellular) that are delivered to acceptor/recipient cells (1). This process profoundly affects key biological activities, including transfer of processed antigen from activated B cells to follicular dendritic cells in the lymph nodes (2), glucose and lipid metabolism via gut-liver communication (3), synaptic activity and plasticity between neurons and glia (4, 5) and at the feto-maternal interface (6). Consequently, alteration or amplification of EV-mediated intercellular communications foster pathophysiological processes (7). Donor and recipient cells may reside in the same microenvironment, in which case EVs regulate paracrine cell-to-cell communication. EVs may also be distributed systemically, via lymph and blood vessels, and operate as endocrine signals between organs or distant cells (8). Although current approaches involving the isolation and injection of exogenous EVs (that is, from cell cultures or biofluids) in animal models permits fine control of pharmacokinetic and pharmacodynamic parameters, it is not clear whether the information obtained from exogenously administered EVs is adequate to address many aspects of EV biology (9). Thanks to their small size and membrane envelope, EVs can

deliver complex and biologically meaningful messages by clustering ligands on their surface and by displaying different signals at once. If EV membranes fuse with recipient cells, EV cargo such as mRNAs and miRNAs is released in the cytoplasm and can extend their biological functions into the recipient cell. However, our knowledge of the cellular and molecular mechanisms that govern cell-cell communication via EVs remains far from comprehensive, at least partly due to technical challenges in tracking and manipulating EVs *in vivo*.

In order to advance the field of EV biology, it is crucial to move beyond exogenous administration of EVs, which incompletely mimics physiological EV release and signaling. Physiological and pathological factors that influence EV composition and function, such as nutrients and 3D cellular architectures, are absent or difficult to recapitulate *in vitro* (10, 11). Moreover, *ex vivo* models in which purified EVs are reinfused intravenously, would allow EV subtypes, some of which would normally act locally, to artificially reach non-physiological sites. For example, EVs involved in ECM deposition and modulation might normally act near the cell of origin (12), as would EVs released at immunological synapses (2, 13). In addition, anatomical differences in vascular permeability (for example, liver versus brain), pathological conditions affecting endothelial barrier function (inflammation and cancer), or defense mechanisms restricting EV diffusion within the draining lymph nodes could alter the biodistribution and cellular targets of EVs (14, 15). Thus, a full understanding of EV signaling will require the study of native, endogenous EVs, defined as EVs released by cells living within a tissue (Figure 1).

The major knowledge gaps in our understanding of native EV (nEV) contributions to intercellular communication can be classified based on their scale: i) at the organ level, the impact of tissue structures and compartmentalization on the biodistribution of EVs; ii) at the cellular level, the significance of the signals delivered to EV recipient cells; and iii) on a molecular level, the mechanistic details of EV-mediated signal transduction.

Biodistribution of EVs is affected by biases from EV isolation

The relative contribution of local vs systemic EV-mediated cell-cell communication is largely unknown. In the last decade, studies aiming at defining where EVs diffuse and accumulate in animal models have employed different EV isolation methods (16). Nonetheless, EV isolation *per se* introduces biases (8, 17) and different EV isolation methods may yield different EV subsets (18). Several investigations reported the impact of EVs in co-culture with different recipient cell types, whether they have or not the ability to come into contact with EVs *in vivo* in the first place. Most *ex vivo* studies have reinfused purified EVs via blood circulation, which brings three separate issues: i) a bolus injection of EVs does not recapitulate continuous or periodic release; ii) the amount of EVs injected is arbitrary and in most studies well above physiological levels; and iii) it is assumed that intravenous reinfusion is the proper biodistribution route, while we and others have demonstrated that nEVs first drain into the lymphatics, and, only after passing the filter of lymph node chains, do nEVs join the systemic circulation (15, 19). For these reasons, in order to understand the *in vivo* biology of EVs, it is crucial to develop approaches to track nEVs under physiological conditions.

Impact of native EVs on recipient cells is unclear

In order to understand the signals that nEVs deliver to target cells, either locally or systemically, it is crucial to determine who are these cellular targets and what impact do nEVs have on them. Given the limited knowledge on EV biodistribution (see point 1), it is unsurprising that the identity of recipient cells targeted by nEVs is also largely unknown. As a consequence, our understanding of the importance of EV-mediated cell signaling is still very rudimentary and mostly derived from artificial model systems. These issues are compounded by the fact that EVs are extremely small [most EV subsets are sub-micron size in diameter (1)], often below the diffraction limit of conventional microscopy (9), and thus, they can carry limited amounts of fluorescent reporters. As a consequence, only recipient cells that bind nEVs in high numbers can be detected and isolated for profiling studies (15, 20). Remote EV-cell communications are much harder to identify as the amount of nEVs exponentially decreases with distance from EV donor cells (21). Therefore, defining the impact of nEVs on the full repertoire of local and distant recipient cells is still an unmet challenge that requires the application of paradigm-shifting technologies (22).

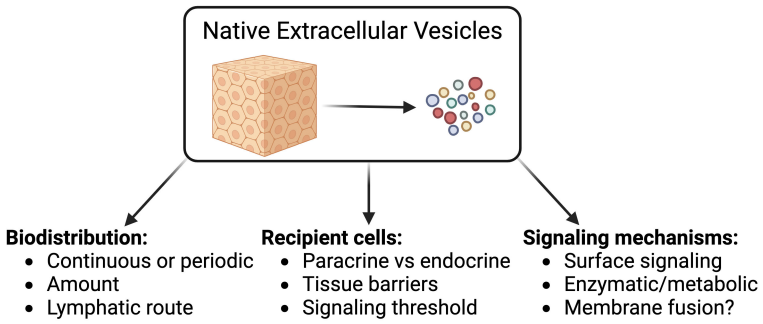

FIGURE 1
Key knowledge gaps in understanding EV biology that can be tackled by studying native EVs.

The mechanisms for EV signal transduction must be validated *in vivo*

Three main mechanisms have been proposed to explain how EVs impact recipient cells. A lot of excitement came from reports describing "horizontal transfer" of bioactive material (including DNA, mRNA and miRNA) between co-cultures of EVs and recipient cells (23). However, evidence of horizontal transfer (or fusion) as a general mode for EV operation *in vivo* is rather scarce, as we and others have reported (15, 17, 24). This is likely due to the fact that endosomal escape is either a rare process or a highly regulated one (25, 26). As a second mechanism, EVs may incorporate active enzymes, which would deliver their enzymatic activity to distant locations (27). A third option is based on classical ligand-receptor interactions between surface proteins and lipids on EVs and transmembrane receptors on recipient cells. In this scenario, EVs represents a key enhancing factor for signaling because they not only allow for clustering of many ligand molecules [which boosts signaling capacity (28)], but they also enable the co-delivery of multiple different signals packaged in the same EV, creating a *de facto* mobile signaling synapse. Lipophilic signaling molecules could similarly be transported via nEV lipid bilayer to alert remote cells (29). It's important to highlight that surface signaling includes mechanisms where components from the extracellular environment bind to nEVs after being released, forming a so-called EV corona (30). The relative contribution of horizontal transfer, enzymatic activity and signaling synapse is largely unclear.

In this context, EVs may represent emerging targets for the prevention and treatment of diseases that stem from environmental exposures (31). This is because EVs are involved in the clearance and transport of proteins and nucleic acids, responding to cellular stress and unwanted molecules (5, 32, 33). Therefore, a better understanding of nEVs is key to improve disease detection and prevention.

Recent progress

Mapping the biodistribution of nEVs

The development of genetic approaches to label and track nEVs promises to revolutionize the field studying EV biology in living organisms (9). We reported for the first time that implanting genetically engineered EV donor cells with bioluminescent EV reporters enables investigations into whole-body biodistribution of nEVs in mice (15). Results from experiments employing this strategy challenged the assumption that tumor-derived EVs directly enter the blood circulation, and instead indicated that lymphatic drainage of nEVs plays an important role in their dissemination (15, 34). These results are consistent with the well-established directionality of interstitial fluid and lymph flow, based on pressure gradients and lymphatic endothelial cell features (35, 36), and suggest that only cell types with access to the systemic circulation (such as endothelial cells) may directly release nEVs into the blood or possibly translocate tissue EVs. The discovery of a lymph-borne biodistribution route for nEVs is foundational for investigations of long-range communication via nEVs, not only in cancer but also during homeostasis and in other conditions, because it maps the barriers encountered by nEVs and short-lists the number of potential recipient cells therein.

Enabling functional studies of nEVs *in vivo*

In order to perform functional studies, a tool to inhibit the release of nEVs is necessary. Such a tool should be specific enough to selectively block nEVs while sparing other soluble factors secreted by EV donor cells. We and others have previously validated expression of Rab35 dominant-negative (DN) mutant (S22N) as a tool to profoundly reduce (>90%) EV release in multiple cell types (15, 37–39). We recently confirmed that expression of Rab35-DN specifically inhibits nEV release, with minimal impact on other secreted factors like cytokines (37). This is important because it enables us to attribute the biological effects of $Rab35^{S22N}$ expression to lack of nEV signaling. The use of a DN mutant allows to avoid the burden of validation that is required when using RNA interference approaches (40, 41). In *in vivo* models of cellular senescence, inhibition of nEV release via expression of $Rab35^{S22N}$ impacted recruitment of specific immune cell types, namely those expressing major histocompatibility class-II surface receptors (37). Coupling this approach with well-established technologies, such as mouse transgenesis or lentiviral vector-based *in vivo* gene delivery (42), promises to open up new frontiers for exploration of nEVs impacts in homeostasis and disease.

Hitting the limits of state-of-the-art approaches

By tracking nEV biodistribution, we discovered that draining lymph nodes were a primary site of nEV accumulation (15). When we employed fluorescent reporters to identify nEV recipient cells at these remote locations, we identified a specialized tissue macrophage as the main recipient cell type (15). These macrophages, located in the sub-capsular sinus of lymphoid organs, were known to capture particulate antigens such as viruses, viral-like particles, bacteria and immune-complexes for initiation of humoral immune responses (eg. antibody production) (43–46). These studies suggest that lymph node B cells may be involved in responding to nEVs signaling. If confirmed, the long-range cross-talk between nEVs and B cells would be the first of its kind to be reported. Understanding the significance of nEV-B cell communication is important to elucidate the influence of humoral immunity during homeostasis and disease (47, 48). Our data support this model, since we detected a significant (albeit modest) increase in nEV binding to lymph node B cells upon depletion of sub-capsular sinus macrophages, and inhibition of nEV release partially reverted the impact of B cells on disease progression (15). However, the signal intensity provided by current genetic EV reporters was not enough to isolate lymph node B cells interacting with nEVs.

Development of next-generation nEV reporters

To increase detection sensitivity for nEV recipient cells, we reasoned that, instead of tagging EVs themselves (first-degree labeling), an approach able to tag recipient cells via nEV binding (second-degree labeling, or "EV painting") would allow us to take advantage of the much larger surface of the recipient cell for reporter accumulation and ultimately would enable isolation and profiling of nEV recipient cells. To this end, we adapted an interaction-based reporter system composed of a transpeptidase enzyme (SortaseA) and its consensus peptide substrate (LPETGS) (22). SortaseA catalyzes the formation of a peptide bond between the consensus peptide and a nearby protein containing an N-terminal glycine residue (49). More than 100 endogenous cell surface proteins contain N-terminal glycine residues in mice, including ubiquitously expressed proteins like histocompatibility antigen receptors and adhesion molecules (50). We selected a SortaseA-based system because it has several advantages over other approaches for studying cell-cell interactions: i) SortaseA labeling is not binary and does not require computational deconvolution, in contrast to PIC-seq (51); ii) SortaseA affinity for its consensus peptide is in the millimolar range and requires *de facto* binding (that is, proximity less than 15 nm between nEV and recipient cell membranes (52), thereby enhancing labeling specificity compared to synNotch system which has nanomolar affinity (53). We designed a membrane-bound form of SortaseA that is seamlessly packaged into EVs, independent of EV donor cell type and without evident alterations in EV biogenesis (22). Upon comparison with a reference EV reporter (CD63-GFP fusion), SortaseA+ EVs generated a signal intensity more than 10-fold higher on EV recipient cells (22). The SortaseA-based nEV reporter allowed us to study cancer stem cell-derived nEVs *in vivo*, within the stem cell niche (54). Future studies using transgenic mice expressing the EV-targeted SortaseA will enable single cell profiling of nEV-recipient cells.

To demonstrate feasibility of using the SortaseA-based nEV reporter *in vivo*, we aimed to demonstrate presence of EV-painted cells in distant organs of mice receiving a skin implant of SortaseA+ nEV donor cells (Figure 2). As expected, nEVs collected in draining lymph nodes, where we detected a strong signal on all B cells, indicating that, at some point, they bound nEVs (Figure 2B). Strikingly, when we analyzed other non-immune organs after perfusion (to remove circulating cells, Figure 2C), we found a significant fraction of lung-resident B cells displaying the mark of interactions with skin nEVs (compare Figures 2D, E). Pulse-chase experiments will address whether these nEV-experienced B cells in the lungs have migrated there from lymph nodes or if they were labeled by circulating nEVs. These data indicate that the SortaseA-based reporter is a viable approach for sensitive detection of long-range cross talk via nEVs. Overall, these results support the idea that nEVs are a type of particulate antigen that signals to B cells located in remote lymphoid organs.

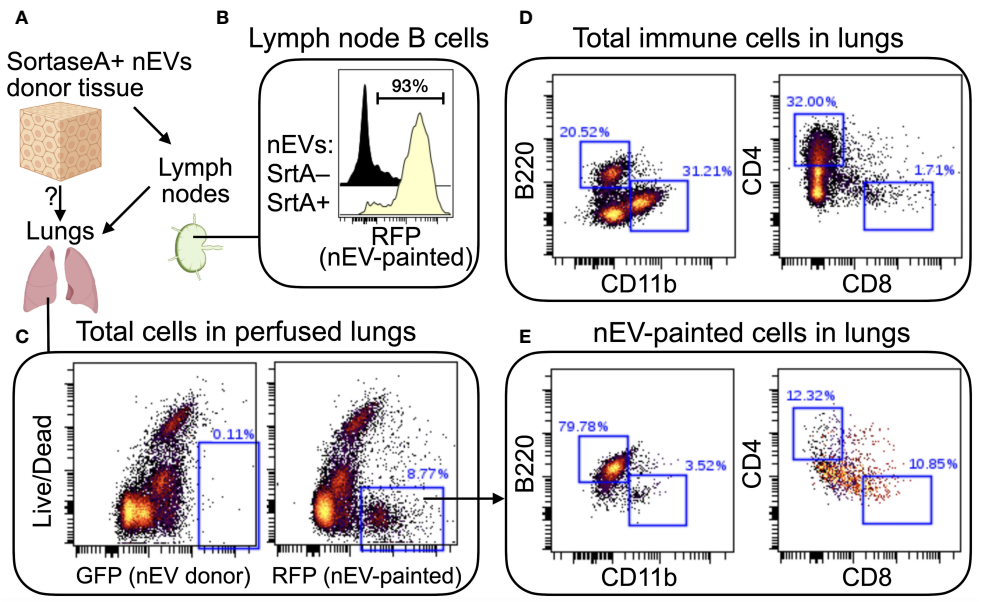

FIGURE 2
In vivo demonstration of long-range cell-cell signaling via nEVs. **(A)** Schematic of the approach. Syngeneic squamous carcinoma cells (MOC2) were engineered to express a membrane-bound form of SortaseA (for inclusion in EV membranes) and to secrete a red fluorescent protein (RFP: mScarlett) tagged with the aminoacid sequence LPETGG (the SortaseA recognition signal). Engineered MOC2 were implanted in the skin of immunocompetent mice (C57B/6). Draining lymph nodes and distant organs, including lungs, were analyzed 21 days later. **(B)** SortaseA+ nEVs accumulated in draining lymph nodes, as expected, where they mainly engaged in cross talks with local B cells. Since tumor cells also express the SortaseA substrate (which is secreted into the extracellular environment and drains into the lymph nodes), when local B cells interact with SortaseA+ nEVs, the nEV-bound enzyme has continuous access to its substrate. Control mice received SortaseA-negative cells. **(C)** Confirmation that nEV-donor cells did not migrate to the lungs (left plot) and that "EV-painted" cells (that is, cells that have experienced nEV binding) were present in perfused lungs. **(D, E)** Comparison of lung-resident total immune cells **(D)** with nEV-painted cells **(E)** highlights a strong enrichment for B cells (B220+CD11b-CD4-CD8-) among the latter. N=2.

Conclusions

There is a case to be made for the study of native EVs, defined as EVs released by cells living within a tissue. The EV community is embracing the importance of studying nEVs to advance our understanding of their biology (55). New biological insights from investigation of nEV in diagnosis of neurodegenerative diseases (56) and cancer (57, 58) are poised to improve prevention strategies. Although innovative approaches that take advantage of nEVs are emerging (59), more studies are needed to unravel the breath of nEV impact in disease. The technologies described in this perspective will support these efforts. Ultimately, coupling advanced EV engineering with mouse transgenesis and modern sequencing technologies will tremendously benefit our understanding of EV biology.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Stanford Cancer Institute. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

DN: Investigation, Writing – review & editing. QL: Funding acquisition, Writing – review & editing. FP: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Benzo[a]pyrene exposure prevents high fat diet-induced obesity in the 4T1 model of mammary carcinoma

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Tumor metastasis is the main cause of death in triple-negative breast cancer (TNBC) patients. TNBC is the most aggressive subtype of breast cancer lacking the expression of estrogen, progesterone, and human epidermal growth factor 2 receptors, thereby rendering it insensitive to targeted therapies. It has been well-established that excess adiposity contributes to the progression of TNBC; however, due to the aggressive nature of this breast cancer subtype, it is imperative to determine how multiple factors can contribute to progression. Therefore, we aimed to investigate if exposure to an environmental carcinogen could impact a pre-existing obesity-promoted cancer. We utilized a spontaneous lung metastatic mouse model where 4T1 breast tumor cells are injected into the mammary gland of BALB/c mice. Feeding a high fat diet (HFD) in this model has been shown to promote tumor growth and metastasis. Herein, we tested the effects of both a HFD and benzo(a)pyrene (B[a]P) exposure. Our results indicate that diet and B[a]P had no tumor promotional interaction. However, unexpectedly, our findings reveal an inhibitory effect of B[a]P on body weight, adipose tissue deposition, and tumor volume at time of sacrifice specifically under HFD conditions.

KEYWORDS

triple-negative breast cancer, obesity, benzo[a]pyrene, high-fat diet, adipose tissue, metastasis

1 Introduction

Obesity is characterized by a state of chronic local and systemic inflammation and exerts significant effects on the development of triple negative breast cancer (TNBC), an aggressive breast cancer subtype that has a high rate of reoccurrence and distant metastasis (1). In obesity, the elevated production of estrogen by adipose tissue affects circulating estrogen levels, and it is widely recognized that these growth-promoting effects of estrogens are essential in the development and progression of human breast cancer. However, estrogen does not have a direct impact on TNBC, since it does not express the estrogen receptor. Metastatic spread of

TNBC to distant sites occurs earlier in patients with obesity leading to higher mortality rates (2). The molecular basis for why triple negative tumors are more aggressive in patients with obesity has been explored (3), but the potential impact of excess adiposity on the response to environmental carcinogenic exposure is not well understood. One study demonstrated that obesity promotes carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA)-initiated mammary tumorigenesis (4). Additionally, epidemiological studies have demonstrated that certain inflammatory conditions can exacerbate carcinogenesis in the context of environmental modifiers. For example, lung cancer is promoted when patients with chronic obstructive pulmonary disease are also cigarette smokers (5). Aflatoxin B exposure synergistically interacts with hepatitis B virus to induce hepatocellular carcinoma (6). Dietary intake of mutagenic compounds in the context of ulcerative colitis promotes colon cancer (7). Herein, we aim to determine if benzo(a)pyrene (B[a]P) exposure promotes lung metastasis in the context of a high-fat diet (HFD), as lung is one of the most common sites of metastatic spread of TNBC (8).

B[a]P is a lipophilic polyaromatic hydrocarbon (PAH) that readily crosses cell membranes and activates the aryl hydrocarbon receptor (AhR) in the cytoplasm. Once activated, AhR is transported into the nucleus where it recognizes and binds to xenobiotic-response elements in gene promoters regulating various PAH-responsive genes. Importantly, AhR target genes include the cytochrome P450 gene family, including *CYP1A1*, and *epoxide hydrolase*, which are able to metabolize B[a]P (9, 10). Once B[a]P is metabolized to BPDE, this metabolite covalently binds to the N2 position of guanines in DNA, forming bulky adducts that can lead to initiating mutations (11, 12). We have previously demonstrated that kynurenine released from adipocytes activates AhR leading to cancer progression *in vitro* (13). How potential endogenous and exogenous AhR ligands may converge to promote cancers has not been explored. We hypothesized that B[a]P enhances the mammary tumor promoting activity of a high fat diet (HFD) leading to tumor progression and metastasis. To test our hypothesis, we subjected the 4T1 transplantation mouse model of TNBC to B[a]P exposure while concurrently feeding a HFD. The host mouse is BALB/c, which expresses the Ah^{b-2} allele. The Ah^{b-1}, Ah^{b-2}, and Ah^{b-3} alleles encode AhRs that bind to aromatic ligands like PAHs with high affinity (14).

2 Materials and methods

2.1 *In vivo*

Female BALB/c mice were purchased from Charles River Laboratories (MI, USA) at six-weeks of age. Mice were randomly divided into high fat (60% kcal fat); or control diet/low-fat diet (10% kcal fat) (Bio Serve Cat# F3282 and F4031) diet groups (*n=10*). Four weeks after starting the mice on diets, 4T1 cells (4.0×10^5 cells) obtained from American Type Culture Collection (ATCC) were suspended in 50 µL Matrigel (Corning Cat# 354248) and injected into the abdominal mammary fat pad of mice. Five days after 4T1 cells injection, mice received intraperitoneal (IP) injections of either sesame oil (Sigma Cat# S3547) carrier only or benzo[a]pyrene (2 mg/kg/body weight, Sigma Cat# B1760) dissolved in sesame oil for 20 days. 4T1 tumors were measured throughout the study and at sacrifice. Mice were sacrificed 26 days after 4T1 cell injection. Mice were weighed, and mammary and visceral fat pads, tumors, and lungs were excised and weighed. Lungs were removed and inflated with PBS via the trachea and placed in 10% neutral buffered Formalin (Azer Scientific, Cat# PFNBF-20) for 72 hours. Lung specimens in 70% ethanol and frozen mammary adipose tissue were provided to the MSU Histology core for hematoxylin and eosin (H&E) staining. Transverse sections were obtained, and slides were prepared for quantification.

2.2 Imaging

Imaging and counting of metastatic sites were performed on an Olympus BX40 microscope. Adipocytes images were analyzed using Image J. Briefly, after importing an image of interest, a known linear scale bar was used to set the scale of the image. The distance of the scale was analyzed to input the distance of the scale bar. The pixel aspect ratio was adjusted to 1, with the unit specified as (µm). Subsequently, background was subtracted, and the images were converted to 8-bit and thresholded to highlight adipocyte areas. Finally, the areas corresponding to adipocytes were measured to quantify adipocytes area. Tissue was randomly sectioned by the histology core and microscopist was blinded to the experimental groups when quantifying lung metastases and adipocyte size.

2.3 Kynurenine ELISA

Serum kynurenine was measured by enzyme-linked immunosorbent assay (ELISA), according to manufacturer's instructions. At sacrifice, blood was collected by cardiac puncture and allowed to clot at room temperature before being centrifuged at 1000 RPM for 5 min. Serum was collected and stored at -20°C. Serum was used for the ELISA to quantify kynurenine levels. Kynurenine ELISA kit was obtained from Immusmol (Bordeaux, France).

2.4 Dietary information

Mice were randomly divided into High Fat (60% kcal fat; 20.5% of protein, 36.0% of fat, 3.5% of ash, 0.0% of fiber, <10% of moisture, and 35.7% of carbohydrate) or Control Diet (10% kcal fat; 20.5% of protein, 7.2% of fat, 3.5% of ash, 0.0% of fiber, <10% of moisture, and 61.6% of carbohydrate) groups (Bio Serve Cat# F3282 and F4031) groups. The nutritional composition is similar between diets except for fat and carbohydrate. Tryptophan is obtained from a

dietary protein source; since protein composition is 20.5% for both diets, the level of tryptophan in each diet is similar. Food consumption was measured and remained stable across experimental groups.

2.5 Statistical analysis

Each bar of tumor size represents the mean ± SEM ($n = 10$). Each bar of body weight represents the mean ± SEM ($n = 10$). The data were evaluated using a two-way ANOVA and were expressed as a mean ± SD. A Tukey's test was used to compare the means of each treatment/exposure to the means of all other treatment/exposure groups. Values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ were considered statistically significant. Statistical analyses were performed using GraphPad Prism version 10 for Mac, GraphPad Software, La Jolla, CA, USA (www.graphpad.com).

3 Results

3.1 B[a]P exposure reduces mammary 4T1 tumor volume in HFD-fed mice

BALB/c 4T1 tumor bearing mice were fed respective diets and exposed to either B[a]P or vehicle treatment for 26 days. The volume of tumor at sacrifice was not significantly larger in LFD-fed compared to HFD-fed mice or LFD-vehicle compared to LFD-B[a]P. However, opposite to what was hypothesized, B[a]P exposure significantly reduced the effect of HFD on tumor volume at time of sacrifice ($p<0.0001$). The number of lung metastases were not affected by diet and/or B[a]P exposure (Figure 1B). Statistical differences in lung weights by diet and/or B[a]P exposure were not detected (Figure 1C). The weights of the kidney, spleen, and liver were not significantly impacted by diet and/or B[a]P exposure, even when normalized to bodyweight (data not shown).

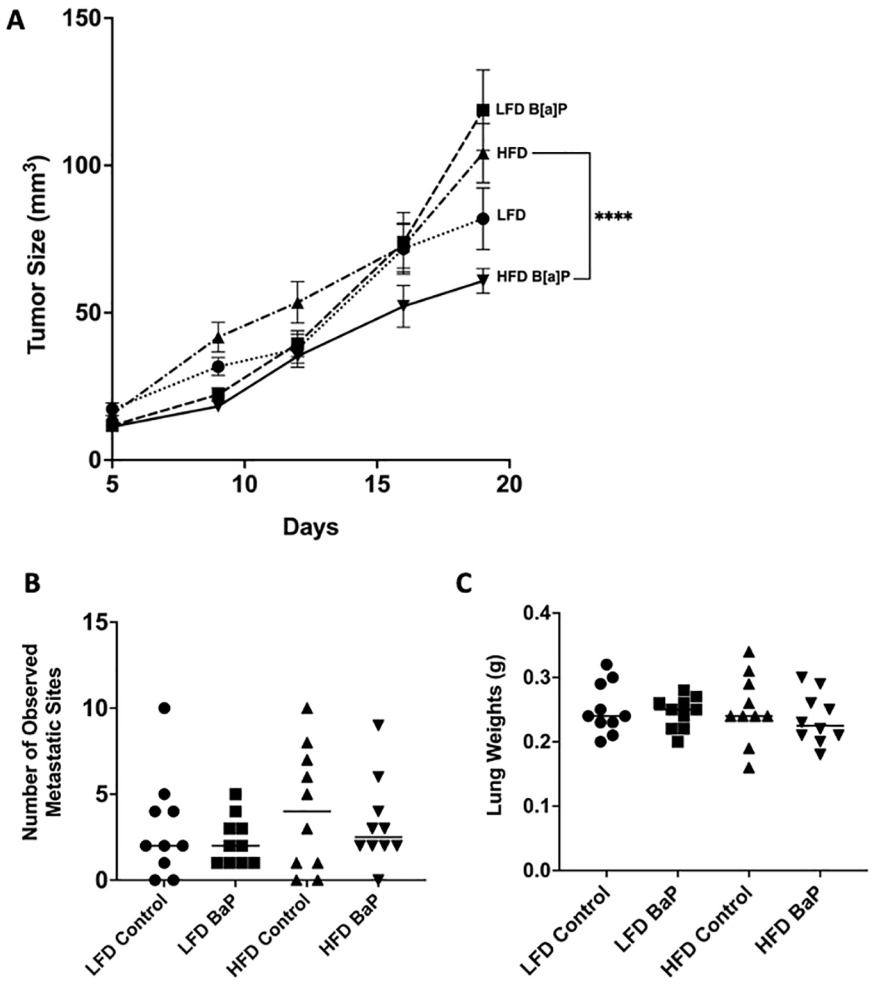

FIGURE 1
B[a]P-exposed mice on a high-fat diet have significantly reduced tumor volume at time of sacrifice compared to vehicle-exposed mice on a high-fat diet. **(A)** Tumor volume over the last 14 days of diet and B[a]P or vehicle treatment. **(B)** Number of observed metastatic sites quantitated under the microscope. **(C)** Lung weights measurements at sacrifice. Each bar represents the mean ± SEM (n = 10 mice/group). A two-way ANOVA was performed to determine statistical significance and the value ****$p < 0.0001$ is considered statistically significant.

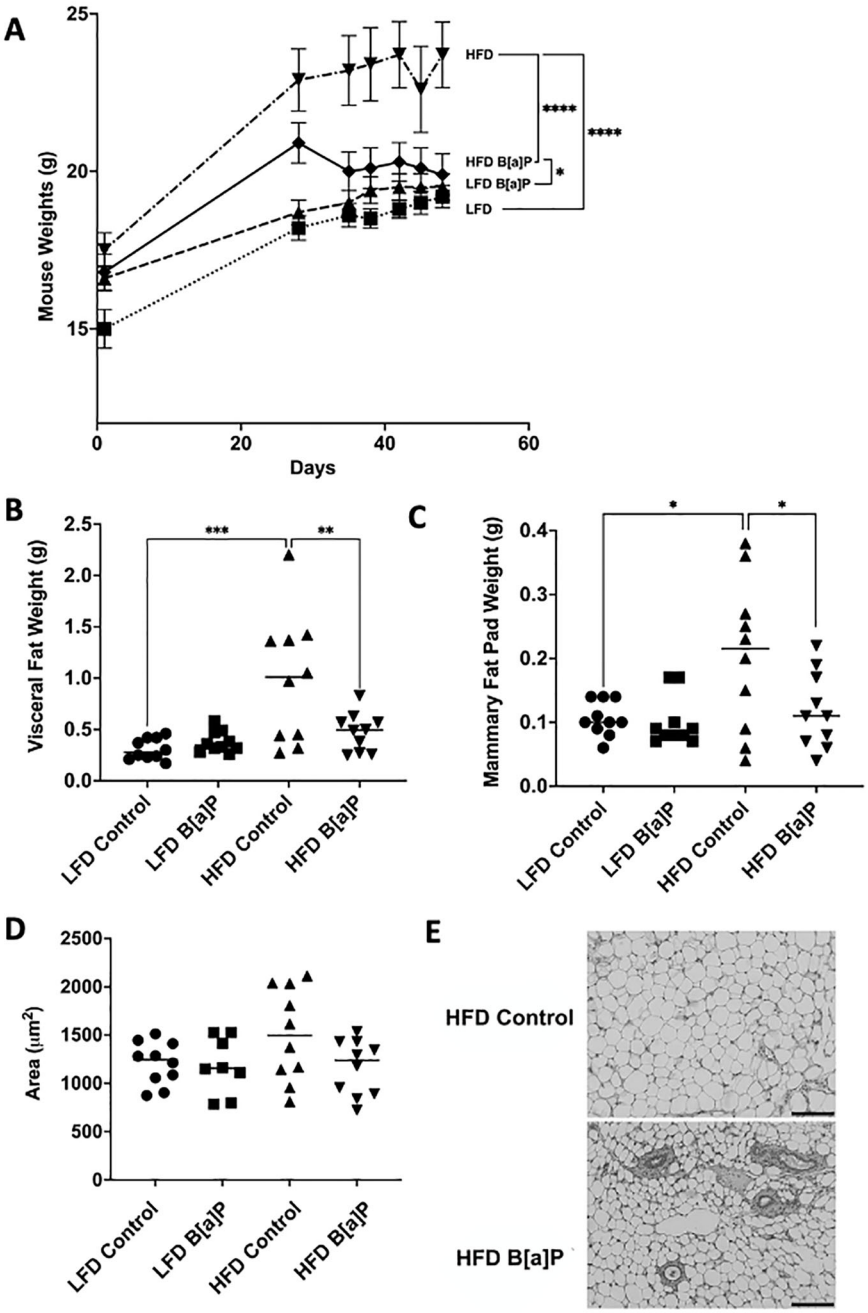

FIGURE 2
B[a]P exposed mice on a high-fat diet have significantly reduced body weight, visceral fat pad weight, and mammary fat pad weight at time of sacrifice compared to vehicle exposed mice on a high-fat diet. **(A)** Mouse body weights over the course of the study. Mice were on respective diets and were administered B[a]P or vehicle. **(B, C)** Visceral and mammary fat pad weights at sacrifice, respectively. **(D)** Analysis of mammary adipocytes size in hematoxylin and eosin in stained mammary fat pad. **(E)** Microscopy of mammary adipose tissue; scale bar 140μm. Each bar of body weight represents the mean ± SEM (n = 10 mice/group). A two-way ANOVA was performed to determine statistical significance and values $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ are considered statistically significant.

3.2 B[a]P reduces body weight in HFD-fed mice

The body weights of mice fed a HFD were significantly greater compared to those of mice fed the LFD at the time of sacrifice ($p<0.0001$) (Figure 2A). The body weights of B[a]P-exposed mice fed a HFD were significantly greater compared to B[a]P-exposed mice fed a LFD at the time of sacrifice ($p<0.05$) (Figure 2A). Interestingly, the body weights of B[a]P-exposed mice fed a HFD were significantly lower compared to vehicle-exposed mice fed a HFD at the time of sacrifice ($p<0.0001$) (Figure 2A). HFD-fed mice had a significantly increased visceral adipose tissue (Figure 2B) and mammary adipose tissue (Figure 2C) weight compared to mice on the LFD ($p<0.001$). Consistent with the reduced total body weight,

HFD-fed mice exposed to B[a]P had a significantly reduced visceral (Figure 2B) and mammary (Figure 2C) adipose tissue weight than HFD-fed, vehicle exposed mice ($p<0.01$). A reduction in average mammary adipocyte size was observed in HFD-fed mice exposed to B[a]P compared to HFD-fed vehicle exposed mice, although not significant (Figure 2D). Images show mammary adipose tissue (Figure 2E).

3.3 Serum kynurenine concentration is significantly increased in HFD-fed mice exposed to B[a]P

Serum kynurenine levels were not significantly different in HFD-fed mice when compared to LFD-fed mice (Figure 3). Serum kynurenine levels were not significantly different between B[a]P or vehicle exposed mice on LFD (Figure 3). Only the combinatorial effect of B[a]P exposed mice on a HFD significantly increased serum kynurenine compared to all other groups. B[a]P exposed mice fed a HFD had significantly higher levels of serum kynurenine compared to B[a]P exposed mice on a LFD ($p<0.001$) or vehicle exposed mice on a HFD ($p<0.05$) (Figure 3).

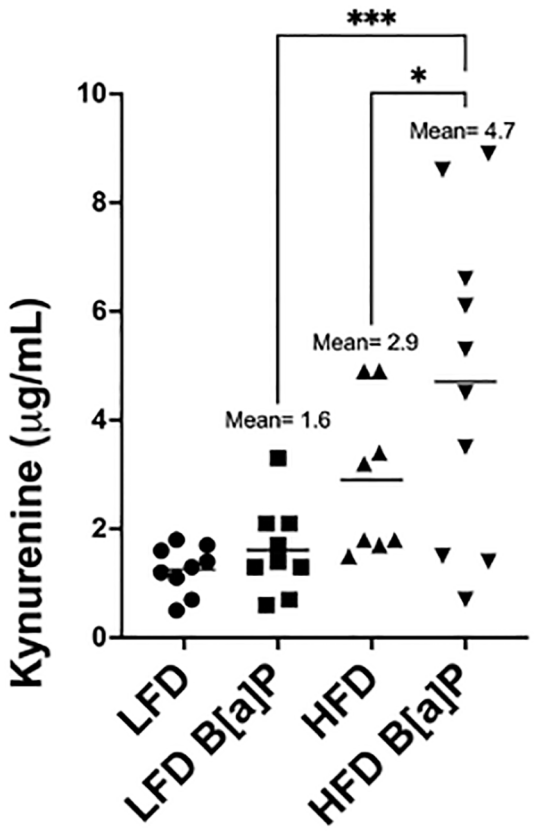

FIGURE 3
Serum kynurenine concentration is significantly increased in HFD-fed mice exposed to B[a]P. Serum kynurenine concentrations as measured by ELISA. A two-way ANOVA was performed to determine statistical significance and values *$p < 0.05$ and ***$p < 0.001$ are considered statistically significant.

4 Discussion

High-fat diets are a major contributor to obesity and a strong risk factor for triple-negative breast cancer (TNBC) (15–19). Herein, the HFD-fed mice developed tumors that were not significantly different in volume compared to those of LFD-fed mice or LFD-vehicle compared to LFD-BaP suggesting no tumor promotional effect of diet or B[a]P, individually in this study. (Figure 1A). This result may be due to the shorter duration of HFD-feeding compared to previously published study which demonstrated that long-term (16 weeks) intake of a high-fat diet increases tumor growth of TNBC cells (4T1 cells) in BALB/c mice (20). Our rationale for only feeding the HFD for 4 weeks prior to B[a]P exposure was based on our hypothesis that there would be an interaction between diet and B[a]P exposure on tumor promotion and metastasis. Herein, we show that B[a]P exposure was associated with reduced body weight, reduced overall mammary and visceral fat weight and reduced tumor volume only when the 4T1 tumor-bearing mice were challenged with a HFD, suggesting that B[a]P inhibited HFD-promoted weight gain. Due to the lipophilicity of polyaromatic hydrocarbons such as B[a]P, bioaccumulation in adipose tissue has been observed in several species (21) and in mammary glands (22). However, the adipose tissue reducing mechanism of B[a]P observed herein is unknown.

B[a]P has a wide range of biological activities. B[a]P interacts with the AhR, induces reactive oxygen species (ROS), forms DNA adducts, promotes immunosuppression, modulates the microbiome, and induces epigenetic changes. Many of these mechanisms have been shown to influence adiposity and/or inflammation. B[a]P can promote hematotoxcity which may induce immunosuppression and impair HFD-promoted inflammatory responses and adiposity (23). Another AhR-independent mechanism may involve the translocation of hormone-sensitive lipase, a critical component of the lipolytic pathway responsible for fat breakdown. This translocation is stimulated by an increase in ROS in adipose tissue, highlighting the essential role of ROS in the complete process of lipolysis (24, 25). Therefore, further exploration of the translocation of hormone-sensitive lipase and its stimulation by ROS in visceral and mammary adipose tissue could contribute to a more comprehensive understanding of the adipose tissue-reducing effects of B[a]P. In addition to impacting adipogenesis, B[a]P could directly influence cancer cells by unknown mechanisms. Other benzo-derivatives have apoptosis-inducing activities in cancer cells. Anti-tumor effects have been identified for benzopyran derivatives (26–28) and a novel benzocoumarin-stilbene hybrid (29).

The gut microbiome has emerged as a key player in adiposity regulation. Certain microbial species have been associated with obesity, while others have been linked to leanness. The microbiome can influence adiposity through various mechanisms, including the production of metabolites that affect energy balance and inflammation. For example, the short-chain fatty acid butyrate has been shown to promote energy expenditure and reduce adiposity (30, 31). Primary findings show that butyl butyrate was significantly altered in human microbiota by B[a]P exposure (32) suggesting that B[a]P leads to changes in the abundance of volatile metabolites in the microbiota which can impact adiposity. Future

studies could aim to identify microbial species impacted by B[a]P exposure, elucidating their specific contributions to energy metabolism, inflammatory responses, and adiposity regulation. For instance, experiments could involve microbial community profiling using high-throughput sequencing techniques to assess changes in species diversity and abundance in response to B[a]P exposure. All these potential mechanisms potentially outweigh that of AhR-dependent effects.

Interestingly, B[a]P-exposed mice on a HFD were protected from obesity closely resembling the phenotype observed in studies where mice are deficient in AhR activity. For example, AhR knockout mice are protected from HFD-induced obesity (33). This effect was only observed when challenged with HFD—no effect on weight gain was observed in LFD-fed mice. Adipose tissue-specific depletion of AhR, protected against diet-induced obesity (34). Consistent conclusions are drawn from alternative models: chemical inhibition of AhR by either α-napthoflavone or CH-223191 protected against Western diet-induced weight gain *in vivo* (35). Therefore, there may be a mechanism whereby B[a]P has AhR inhibitory activity by either *1)* reducing the activity of endogenous agonists from adipose tissue, such as kynurenine, and/or *2)* inducing the expression of the AhR repressor.

Several conclusions can be drawn from this study to be applied to future experiment design. Although our prior studies demonstrated that HFD-feeding of mice significantly increased adipose tissue kynurenine (36), herein, circulating kynurenine concentration was not associated with overall adipose tissue weight. The highest concentrations of serum kynurenine were observed in HFD-fed, B[a]P exposed mice (Figure 3)—a group that had significantly less body weight and adipose tissue weight compared to the HFD-fed, vehicle exposed mice. The elevated concentration of serum kynurenine in B[a]P exposed mice on a HFD may be more reflective of the amount of total body AhR activation and/or tumor AhR activation, as the transcriptional activity of AhR induces indoleamine 2,3-dioxigenase (IDO), the enzyme responsible for the catabolizing tryptophan to kynurenine. Elevated IDO leads to increases in kynurenine in a positive feedback manner (37). Tumor cells exhibit a high demand for nutrients, particularly tryptophan, to promote their growth and are capable of releasing kynurenine into the periphery (37). Therefore, elevated levels of serum kynurenine in the B[a]P-exposed group fed a HFD may be linked to the secretion of kynurenine from tumors. To further explore these findings, future studies could test this hypothesis in different mouse strains that have higher and lower affinity AhR alleles to see if we observe the same modulatory effect with respect to kynurenine levels and/or adipose tissue mass in B[a]P-exposed mice challenged with HFD.

We had originally hypothesized that there would be a positive interaction between B[a]P and HFD on progression and metastasis. One study limitation is that 4T1 model of mammary carcinoma is highly aggressive, potentially missing a more sensitive window for detecting interactions. Our hypothesis may be better suited to be tested in transgenic models where tumor formation occurs over the course of months instead of weeks. However, by utilizing the 4T1 model of tumorigenesis, we determined there was no additional effect of B[a]P on the promotion of established cancer. Instead, we unexpectedly discovered that B[a]P had an off-target effect, inhibiting HFD-promoted adipogenesis.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Michigan State University Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

RG: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Validation, Writing – original draft, Writing – review & editing. JB: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Key characteristics of carcinogens meet hallmarks for prevention-cutting the Gordian knot

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The complexity of cancer requires a comprehensive approach to understand its diverse manifestations and underlying mechanisms. Initially outlined by Hanahan and Weinberg in 2000 and updated in 2010, the hallmarks of cancer provide a conceptual basis for understanding inherent variability in cancer biology. Recent expansions have further elucidated additional hallmarks, including phenotypic plasticity and senescent cells. The International Agency for Research on Cancer (IARC) has identified the key characteristics of carcinogens (KCCs) to evaluate their carcinogenic potential. We analyzed chemicals of concern for environmental exposure that interact with specific receptors to induce genomic instability, epigenetic alterations, immune suppression, and receptor-mediated effects, thereby contributing to chronic inflammation. Despite their varying degrees of carcinogenicity, these chemicals have similar KCC profiles. Our analysis highlights the pivotal role of receptor binding in activating most other KCCs, underscoring their significance in cancer initiation. Although KCCs are associated with early molecular or cellular events, they do not encompass processes directly linked to full cellular malignancy. Thus, there is a need to integrate clear endpoints that anchor KCCs to the acquisition of a complete malignant phenotype into chemical testing. From the perspective of toxicology and cancer research, an all-encompassing strategy that incorporates both existing and novel KCCs and cancer hallmarks is essential to enable the targeted identification of prevalent carcinogens and facilitate zone-specific prevention strategies. To achieve this goal, collaboration between the KCC and cancer hallmarks communities becomes essential.

KEYWORDS

KCCs, cancer hallmarks, chemical carcinogens, cancer process, regulatory toxicology, precise toxicology, environmental exposure, key carcinogens characteristics

1 Introduction

Cancer cells interact with a complex microenvironment, underscoring the inherent variability in cancer. Hanahan and Weinberg captured this complexity by outlining the hallmarks of cancer in 2000 and updating them in 2010 (1, 2). Senga and Grose expanded the hallmarks of cancer in 2021 by introducing additional hallmarks, such as dedifferentiation/transdifferentiation, epigenetic dysregulation, altered microbiome, and altered neuronal signaling (3). In 2022, Hanahan proposed the unlocking of phenotypic plasticity, non-mutational epigenetic reprogramming, senescent cells, and polymorphic microbiomes as additional hallmarks and emerging characteristics (4)

The *exposome concept* was introduced as a new paradigm for understanding and measuring all non-genetic factors that influence individuals throughout their lives, serving as a counterpart to the genome (5) This concept underscores the importance of capturing diverse environmental exposures, including chemical, biological, and psychosocial factors, to comprehensively assess their collective impact on health outcomes, such as carcinogenesis (5, 6). The Halifax Project Task Force, a pioneering effort in 2013, employed the original eleven hallmarks to evaluate the carcinogenic potential of environmental chemical mixtures and low-dose exposures (7). This initiative highlighted the critical need for robust data on environmental toxin exposures to elucidate their role in cancer development. Concurrently, an expert panel workshop convened by the International Agency for Research on Cancer (IARC) identified for over hundred Group 1 cancer hazards ten key characteristics of carcinogens (KCCs), offering a structured and comprehensive approach to identify, assess and classify the carcinogenic potential of various environmental agents (8, 9) (Table 1).

The IARC Preamble, as amended in 2019 (10), and the upcoming updated Handbook of the Report on Carcinogens (RoC) by the US National Toxicology Program (NTP (11) to develop monographs underscores the importance of refining current methods and/or adding novel methods to mechanistically identify and assess cancer hazards. This emphasizes the need of utilizing an integrated approach that prioritizes causation over association and incorporates both KCCs and cancer hallmarks for precision environmental health.

To enhance the understanding of the relationship between KCCs and cancer hallmarks, we analyzed a group of chemicals of environmental concern These were classified into different ranks of carcinogenicity by the IARC (12) and NTP (13) including polycyclic aromatic hydrocarbons (PAHs), perfluoroalkyl substances (PFASs), phthalates, and endocrine disruptors (EDC), such as organophosphate (OPFRs) and halogenated (HRF) flame retardants, with arsenic as a paradigmatic representative of heavy metals (Supplementary Table S1).

PAHs and their nitro-derivatives (NPAHs) are among the most significant air pollutants and are implicated in respiratory pathologies, including cancer. PFASs are associated with a spectrum of health concerns, including potential adverse effects on immune function and metabolism. Their carcinogenic properties have recently been revaluated based on new data and KCCs (14).

Phthalates have emerged as significant environmental contaminants, potentially linked to the escalation of various health issues, including reproductive disorders. OPFRs and HRFs are persistent pollutants used to reduce the flammability of various materials including plastics, textiles, and foam products. Some OPFRs such as tris(2,3-dibromopropyl) phosphate (TDBPP) are considered potential human carcinogens. Arsenic is recognized as a significant environmental hazard and is implicated in a range of health issues including cancer. These substances interact with significant receptors, whose activation can initiate molecular events in the carcinogenesis process. These receptors include the aryl hydrocarbon receptor (AhR), peroxisome proliferator-activated receptors (PPARs), estrogen and/or androgen receptors (ERs; AR), thyroid hormone receptors (THRs), and glucocorticoid receptors (GR).

TABLE 1 Key characteristics of carcinogens and evolution of cancer hallmarks from 2000 to 2022.

Key Carcinogens Characteristics (Smith et al, 2016)
1. electrophilic or can be metabolically activated
2. Is genotoxic
3. Alters DNA repair or causes genomic instability
4. Induces epigenetic alterations
5. Induce oxidative stress
6. Induces chronic inflammation
7. Is immunosuppressive
8. Modulates receptor-mediated effects
9. Causes immortalization
10. Alters cell proliferation, cell death or nutrient supply
Cancer Hallmarks (Hanahan and Weinberg, 2000)
– Self-Sufficiency in Growth Signals
– Insensitivity to Antigrowth Signals
– Evading Apoptosis
– Limitless Replicative Potential
– Sustained Angiogenesis
– Tissue Invasion and Metastasis
– Genome Instability
Cancer Hallmarks: the next generation (Hanahan and Weinberg, 2011)
– Genomic instability and evolution
– Tumor-Promoting Inflammation
– Reprogramming Energy Metabolism
– Evading Immune Destruction
Cancer Hallmarks: the new testament (Senga and Grose, 2021)
– Dedifferentiation/Transdifferentiation
– Epigenetic dysregulation
– Altered microbiome
– Altered neuronal signaling
Cancer Hallmarks: new dimensions (Hanahan, 2022)
– Unlocking Phenotypic Plasticity
– Nonmutational Epigenetic Reprogramming
– Polymorphic microbiomes
– Senescent cells

2 Electrophiles or metabolically activated toxins that induce stemness

The KCC concept identifies electrophiles and metabolically activated toxins. Electrophiles, characterized by their electron deficiency, react with nucleophiles through covalent bonding and form adducts with vital cellular macromolecules, such as DNA. This interaction is central to carcinogenesis. While some carcinogens act directly as electrophiles, others undergo transformation into reactive metabolites by enzymes, such as cytochrome P450s, becoming potent carcinogens (15, 16).

PAHs and NPAHs require metabolic activation to generate electrophilic products that can form DNA adducts. AhR orchestrates the bioactivation and detoxification of activated metabolites. Beyond a certain threshold, detoxification capacity is overwhelmed and adaptive responses become maladaptive, implicating disrupted immune and metabolic pathways in genetic instability (17, 18). Therefore, electrophilicity not only contributes to genomic instability, but also influences the immune response and metabolism.

Both PFASs and some phthalate metabolites are considered electrophilic (19, 20) and have the potential to covalently bind to cellular macromolecules. The electrophilic nature of PFAS has been a topic of debate. While some experts argue that PFAS are not classically electrophilic due to their strong carbon-fluorine bonds and stability ((19), accumulating evidence suggests that PFAS can undergo oxidation/reduction reactions, leading to the formation of reactive intermediates. These intermediates may interact with nucleophilic sites in biological molecules, emphasizing the potential for oxidative stress and its implications in carcinogenesis (21–23). PFAS compounds exhibit different binding behaviors depending on their carbon chain lengths and functional groups. For instance, new classes of PFAS that feature shorter chains and incorporate oxygen molecules are considered to be more reactive.

OPFRs are electrophilic compounds that react with nucleophiles in biological systems (24). As a compound containing bromine atoms, TDBPP can act as an electrophile by seeking electron-rich species to form covalent bonds.

Arsenic is commonly found in the environment as oxides, existing in trivalent or pentavalent forms, with a high affinity for electron-rich groups in biological molecules, such as thiols in detoxification pathways. This disrupts cellular processes and contributes to carcinogenesis and genotoxicity (25).

3 Can cause genomic instability

Genotoxic substances that inflict DNA damage do not always directly result in mutations, raising the question about their classification as carcinogens. Such damage can take various forms, including DNA adducts, strand breaks, or base modifications, which differ fundamentally from mutations that alter the DNA sequence itself, often as a byproduct of repair processes. This distinction underscores the importance of considering genotoxicity alongside the capacity to disrupt DNA repair mechanisms or induce genomic instability as a critical characteristic of carcinogens, aligning with the cancer hallmark of genomic instability. The intersection of genotoxic and mutagenic properties of carcinogens recognized by the IARC (12) and NTP (13) suggests the relevance of these agents in precipitating cancer, especially when considering individuals with a predisposition to genomic instability, such as those with hereditary syndromes that heighten vulnerability to additional environmental insults (26). This aligns with Knudson's two-hit hypothesis (26)., which posits that the path to malignancy often requires multiple genetic insults, highlighting the complexity of cancer development and potential role of environmental toxins in precipitating germline mutations.

The linkage between hereditary cancer syndromes and genomic instability, whether chromosomal (CIN) or microsatellite (non-CIN), through mutations in DNA repair genes exemplifies the intricate relationship between genetic predisposition and cancer risk. (27). For instance, mutations in mismatch repair genes in Lynch syndrome or biallelic mutations in the MYH gene are associated with base excision repair pathways, leading to an elevated rate of G•C to T•A transversions and underlining the critical impact of DNA repair fidelity on cancer susceptibility (28)

Moreover, the role of oxidative stress induced by various carcinogens in contributing to genomic instability further emphasizes the need for a nuanced understanding of carcinogenesis. Oxidative stress can precipitate DNA damage, leading to genomic instability and the accumulation of mutations that facilitate cancer progression by enabling cells to acquire additional malignant traits. Thus, the identification of genotoxic agents and those inducing oxidative stress in genes crucial for DNA damage recognition, repair initiation, or damage prevention, is essential in the context of carcinogenesis. DNA double-strand breaks (DSBs) or interstrand crosslinks have been identified as contributing to an increased susceptibility to a spectrum of cancers, including, but not limited to, breast and ovarian cancer, leukemia, and lymphomas (29–31) Moreover, mutations in genes associated with nucleotide excision repair pathways have been implicated in predisposing individuals to skin cancer (32). There is an acute need to screen for agents specifically causing genomic instability in gatekeeper genes, which play a central role in maintaining genomic integrity.

In addition to their direct interactions with DNA and mutagenic effects, PAHs can induce genetic instability by producing reactive oxygen and nitrogen species (ROS/RNS), which can damage cellular components such as lipids, proteins, and DNA. PAHs also disrupt cell antioxidant defense mechanisms, including the depletion of antioxidant molecules such as glutathione and the inhibition of enzymes such as superoxide dismutase and catalase (33).

PFASs, phthalate metabolites, such as mono(2-ethylhexyl) phthalate (MEHP), and OPFRs can induce oxidative stress through the direct generation of ROS/RNS, or by inhibiting mitochondrial function, leading to an imbalance between ROS production and antioxidant defense mechanisms (34–36). Additionally, they can deplete cellular antioxidants such as glutathione and disrupt antioxidant enzyme activity (36).

Arsenic, particularly in its interconverted forms arsenite (As^III) and arsenate (As^V), undergoes redox reactions within cells, leading to the generation of ROS and subsequent oxidative stress. Arsenite, in particular has been identified as a potent inducer of oxidative stress through mechanisms such as mitochondrial dysfunction and the inhibition of antioxidant enzymes. These effects result in oxidative damage to cellular components, contributing to cellular dysfunction and toxicity (37).

Therefore, all these chemicals considered can induce genetic instability by generating oxidative stress, thereby fostering inflammation (38).

4 Induces epigenetic alterations

The rapid assessment of epigenetic effects is essential for both short-term and long-term consequences of toxin exposure, considering that the KCCs and hallmarks both propose epigenetic dysregulation and non-mutational epigenetic reprogramming (39). Studies on non-smoking Polish coke-oven workers exposed to PAHs found alterations in DNA methylation, including increased global and IL-6 gene methylation, and reduced methylation of p53 and HIC1 tumor suppressor genes. p53 hypomethylation is linked to chromosomal instability and higher micronuclei levels, suggesting that DNA methylation modifications are potential biomarkers of cancer risk due to PAH exposure (40).

PFASs can induce epigenetic alterations associated with childhood cancers, such as ependymomas (19, 41, 42). When combined with a high-fat diet, PFASs can support prostate cancer progression through epigenetic, transcriptomic, and metabolomic alterations, indicating a complex interplay between metabolism and epigenetics during cancer development (43, 44).

Phthalate exposure can alter DNA methylation and miRNA production and induce transgenerational epigenetic changes that affect transgenerational disease susceptibility (45, 46).

OPFRs exposure is associated with alterations in DNA methylation patterns and histone modifications (47).

Arsenic metabolism involves methylation reactions that share similarities with DNA methylation pathways, suggesting a potential interplay between arsenic metabolism and DNA methylation. Arsenic exposure can cause global changes in DNA methylation, and is associated with prostate cancer (48, 49).

Epigenetic changes can affect receptors and trigger molecular events that may cause cancer. DNA methylation patterns can silence or alter receptor gene expression, thereby affecting normal signaling. Histone modifications can change the chromatin structure and influence receptor expression by impacting promoter accessibility. mRNAs can regulate receptor expression by targeting messenger RNAs to degrade or inhibit their translation. Although some EDCs may disrupt epigenetic programming during development, it remains unclear whether this leads to negative outcomes (50) This emphasizes the pivotal role of receptor-mediated effects in the context of KCCs, highlighting how epigenetic changes contribute to receptor dysfunction and subsequent carcinogenic processes.

5 Induces chronic inflammation

Chronic inflammation, a key characteristic of carcinogenesis, is intricately linked to the hallmarks of tumor-promoting inflammation and alterations in the microbiome or polymorphic microbiomes. This association is due to both the direct effects of environmental toxins and indirect effects via changes in the microbiome at the population level.

5.1 Direct impact on immune-inflammatory responses

Research integrating single- and multiple-exposure models has shed light on the immunoinflammatory response to mixed chemical exposure, revealing the differential effects of chemicals on immune-inflammatory markers.

All chemicals that induce oxidative stress are potentially involved in the inflammatory processes.

The immune-inflammatory response to environmental exposure is mediated by AhR activation, leading to inflammasomes and adaptive responses. Chronic inflammation occurs when adaptive responses become maladaptive due to sustained or high exposure (17, 18).

PAHs and metals have been identified as significant influencers, underscoring the complexity of the health effects of multiple chemical exposures (41, 51) necessitating the development of sophisticated models to decipher the interactions and non-linear relationships between chemical co-exposure and immune-inflammatory responses.

5.2 Indirect impact through the microbiome

The ubiquitous Helicobacter pylori, co-evolved with humans for 50,000 years, represents the dual role of microbiota in health and disease. H. pylori is associated with a reduced risk of certain diseases and interacts with gut microbiota to influence metabolic processes (52, 53). However, its presence has also been implicated in a significant proportion of gastric cancers (54).

The microbiome impact on cancer progression supports Paget's seed and soil hypothesis. The microbiome can alter the tumor microenvironment, thereby facilitating or hindering cancer development. This corresponds to the discovery that oncoviruses, such as the Rous sarcoma virus, necessitate a suitable "soil" for the oncogenic "seeds" to thrive (55–57).

Chronic inflammation, tumor-promoting inflammation, and altered microbiomes are key factors to consider when determining the environmental toxins that drive precursor lesions to malignancy. These factors exploit the extended latency period, as seen in colorectal cancer development (58), to prevent such progression.

Environmental pollutants, such as heavy metals, pesticides, and food additives, can harm gut microbiomes and potentially cause or exacerbate human diseases. This damage can result from both direct

and indirect effects on gut bacteria, leading to alterations in the microbial diversity and metabolic processes.

One mechanism of microbiome toxicity is the changes in the microbial metabolites, which bind AhR or t (59) he Farnesoid X Receptor (FXR) (60), affecting the immune response and metabolism.

PFAS can lead to alterations in gut microbiota and reduce microbiome diversity (61, 62).

DEHP modifies mouse intestinal microbiota, affecting metabolism and intestinal integrity (63).

Arsenic affects microbiome composition and function, with microbial redox transformations influencing its fate and toxicity when inhaled or ingested (64–66).

6 Is immunosuppressive

Cancer immune evasion and immunosuppression have distinct, yet interrelated mechanisms and implications.

Immune evasion, a cancer hallmark, refers to tumor's ability to avoid immune detection through various strategies, including immunosuppression. In metastatic melanoma treatments, such as adoptive cell transfer therapies, initial remission can be followed by relapse due to melanoma cell dedifferentiation influenced by proinflammatory cytokines such as TNF-α within the tumor microenvironment, facilitating immune evasion through antigen loss (67).

The distinction between immunosuppressive effects, such as those observed in organ transplantation, and immune evasion strategies, including camouflage of the immune system, prompts a revaluation of this key characteristic. Environmental toxins, while traditionally associated with immunosuppression, may also play roles in facilitating immune evasion, underscoring the need for a broader focus on environmental factors that hinder the body's immunosurveillance mechanisms against cancers.

The role of AhR in both cancer immune surveillance and immune evasion makes it a potential target for disruption and tumor promotion by exogenous chemicals, such as PAHs (68).

PPARs regulate immune responses by controlling inflammation and immune cell activity. AhR, PPARs, and other nuclear receptors interact to enhance immunosuppression.

PFAS have emerged as a significant concern because of their potential to induce immunosuppression (69).

Other environmental contaminants, ranging from fungicides and herbicides to personal care substances and industrial agents such as DEHP, which affect cytokine secretion (70), have been implicated in potentially compromising tumor immunosurveillance.

7 Modulates receptor-mediated effects

The nexus between receptor-mediated signaling and perpetuation of cell proliferation underscores a fundamental aspect of carcinogenesis, emphasizing the critical role of receptor pathways in the broader context of cancer cell growth and beyond molecular initiating events. Many chemicals, such as PAHs, PFAS, and phthalates, interact with multiple receptors, leading to complex downstream effects. Recent reviews provide a broader spectrum of receptor-mediated pathways involved in these interactions (71–73).

An integrated approach to understand how the modulation of receptor-mediated pathways directly contributes to the characteristics of sustained proliferative signaling is crucial.

PFASs underscore the connection between receptor modulation and proliferative signaling because of their ability to act as PPARs agonists or antagonists.

Tetrabromobisphenol A (TBBPA), a widely used HFR, interacts with both ER and AR, leading to a combined effect (74), which has been proposed as a mechanism in the carcinogenesis of triple-negative breast cancer (TNBC) (75), and with THR (76, 77), modulating genes involved in thyroid cancer, through epigenetic alterations (77, 78).

Arsenite can impede GR-mediated transcription at non-toxic levels, impacting nuclear function without affecting hormone-induced activation or translocation (79) Other chemicals, such as benzophenone-1 (BP1), affect ER pathways, which regulate cell proliferation and cell cycle. BP1 stimulates ER-positive cancer cells and modulates cyclin D1 expression, highlighting the importance of these pathways in maintaining proliferative signaling (80).

Taken together, these insights argue for a paradigm that acknowledges that screening for environmental toxins that mediate receptor-mediated modulation must essentially focus on sustained proliferative signaling as one of the majors read out (81).

8 Causes immortalization: altered lengthening of telomeres & evasion of cell death

The focus on immortalization and evasion of apoptosis, two critical hallmarks, contributes significantly to the understanding of environmental toxin-driven cancer via KCCs.

Telomere dynamics, influenced by environmental factors such as bisphenol A (TBBPA) and persistent organic pollutants (POPs), underscores the role of toxins in aging and disease susceptibility (82, 83). Investigations of telomere alterations among astronauts and arsenic exposure further highlight the complex interplay between toxins and telomere length regulation (84, 85).

Disrupted regulation of apoptosis by environmental mutagens, including endocrine-disrupting chemicals (EDCs) such as bisphenol A, is implicated in cancer development (86). Climate change exacerbates this risk by altering environmental stressors, contributing to air pollution complexity, and disrupting the apoptotic signaling pathway (87).

PAHs have been implicated in oral squamous cell carcinoma by influencing cell fate decisions and promoting cell immortalization during senescence (88).

Exposure to PFAS has been associated with altered plasma membrane fluidity, affecting calcium signaling and increasing platelet response to agonists, potentially influencing cell survival and evasion of cell death (89).

High arsenite concentrations may decrease telomerase activity and telomere length, leading to apoptosis (90).

9 Deregulating cellular energetics: a fulcrum for KCC - alters cell proliferation, cell death, or nutrient supply

The final key characteristic of carcinogens, instead of focusing on three different aspects, which have already been addressed via other characteristics, may benefit from specifically focusing on deregulating cellular energetics. This focus will be crucial for mitigating toxin exposure and cancer metabolic underpinnings.

PAHs offer a good example of how chemical exposure can deregulate cellular energetics, highlighting the intricate relationship between environmental toxins and cancer metabolism. PAHs can induce metabolic reprogramming by generating ROS and causing mitochondrial dysfunction. This leads to a shift towards glycolysis and away from oxidative phosphorylation, creating an environment favorable to cancer cell growth and survival (91). PAHs reactive intermediates such as diol epoxides can bind proteins leading to the generation of advanced glycation end products (AGEs) through the Maillard reaction (92). AGEs, interacting with their receptors (RAGE), induce metabolic disruption and histone glycation and trigger. the activation of key inflammatory signaling pathways (93). This mechanism has been confirmed in occupational exposure to PAHs, and metal fumes in shipyard welders (94).

PFAS are implicated in inducing epigenetic alterations and influencing cell proliferation, potentially contributing to cancer development (19).

Phthalates are associated with the redox control of cancer cell destruction, where factors such as insufficient oxygen and nutrients can lead to cell death in tumor masses (95).

OPFRs affect diverse molecular pathways controlling cell proliferation and death, potentially contributing to cancer development (96).

Arsenic exposure has been linked to alterations in the gut microbiome, which can influence nutrient supply and potentially contribute to cancer development (97).

10 Discussion

KCCs are intrinsic properties of chemical molecules that contribute to carcinogenesis initiation and sustenance. However, assessing whether these characteristics lead to adverse carcinogenic effects depends on factors such as the exposure concentration, immune system integrity, and tissue-specific response variability (17, 98, 99) (Chemical molecules may exhibit multiple characteristics depending on factors such as the exposure route and the target organ. This complexity underscores the need to consider the interrelationships among KCCs and between KCCs and cancer hallmarks.

We examined chemicals previously evaluated for their potential to cause cancer and found that although the evidence of carcinogenicity varies in strength, they share similar KCCs. Our analysis r suggested that receptor activation is a primary molecular event driving the mechanisms supported by KCCs. Receptor binding plays a crucial role in activating other KCCs, including electrophilicity, immune response disruption, oxidative stress, and inflammation, highlighting the connections between KCCs and their role in cancer initiation.

This complexity highlights the need to study the links between KCCs and cancer hallmarks, investigating how chemical molecule

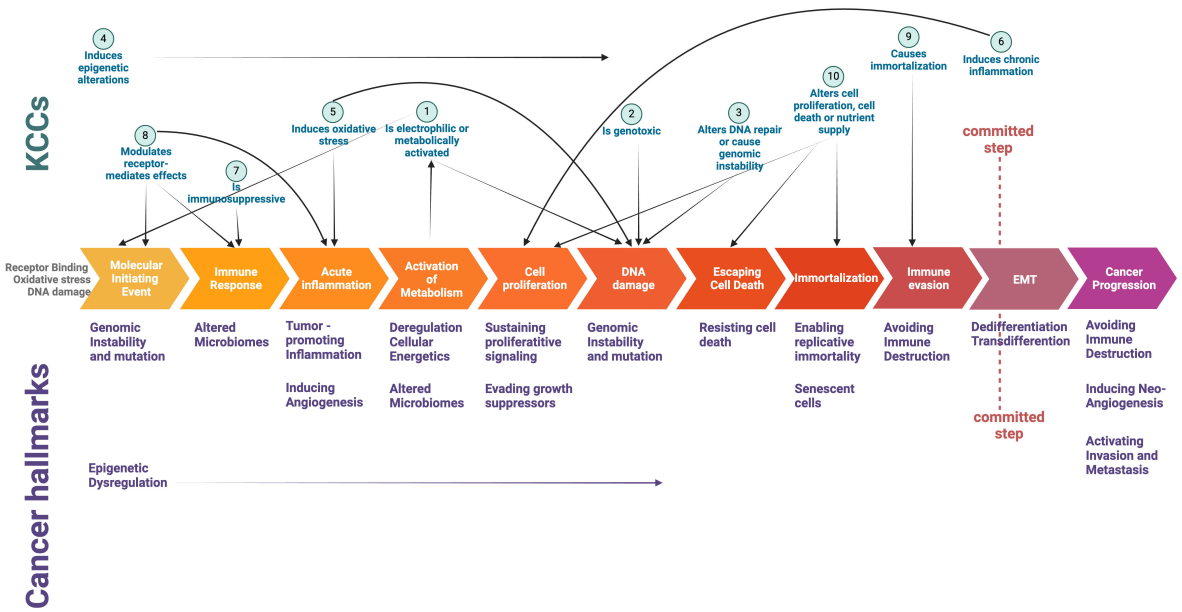

FIGURE 1
Intersection of Key Characteristics of Carcinogens (KCCs) and Cancer Hallmarks in a schematic depiction of critical events underlying cancer initiation and progression. The committed step indicates the critical transition towards a full malignant phenotype (see ref #73). *Created with Biorender.com*.

behavior aligns with hallmark responses (e.g., pathway analysis for common downstream events, multi-omics and spatial data analysis combined with phenotypic activity). The OECD Integrated Approach for Testing and Assessment (IATA) for non-genotoxic carcinogens integrates multiple sources of information and data to assess the carcinogenic potential of a substance and incorporates cancer hallmarks as part of the evaluation process of chemical substances (98, 99). It is rooted in the Adverse Outcome Pathway (AOP) framework, to use key events and molecular pathways in carcinogenesis to pinpoint chemical targets crucial for sustaining cancer progression (98, 99). From this perspective, KCCs appear to be associated with early events that manifest at the molecular or cellular level, but do not involve processes more directly linked to cellular transformation towards malignancy, such as dedifferentiation or transdifferentiation, and the plasticity stemming from cytoskeletal rearrangement to epithelial-mesenchymal transition (Figure 1).

11 Perspective

While KCCs may relate to a chemical's potential to initiate the carcinogenesis process, they do not encompass the cellular context's ability to adapt, show resilience, or mount defense mechanisms. Examining the transition from adaptive to potentially harmful responses could offer further clarity in assessing carcinogenic risks.

To ensure the accuracy of testing, it is important to incorporate the ability to identify malignancy (81, 100). For instance, microenvironment changes can help identify the early influences of carcinogens in promoting tumorigenesis (81, 101, 102) and serve as biological markers of chemical exposure (103). This ensures a comprehensive evaluation that not only identifies the initial stages of carcinogenesis, but also captures the ultimate endpoint of malignancy, providing a system toxicology-oriented and more holistic understanding of the carcinogenic potential of the tested chemicals. This aligns with the Carcinogenicity Health Effects Innovation Program's goal (104), part of the new NIEHS FY 2025-2029 Strategic Plan, of creating a deeper understanding of the mechanisms through which environmental exposures affect biological processes leading to cancer disease (104).

The cancer hallmarks have greatly improved our knowledge of the mechanisms underlying cancer. However, toxicologists require a comprehensive framework that not only identifies carcinogenic agents using the KCCs, but also incorporates the intricate principles of cancer hallmarks. This integration is crucial for creating a robust methodology that can proactively detect potential carcinogens.

Targeted identification of prevalent carcinogenic toxins can be facilitated by integrating the KCC concept with hallmark-based mechanisms, thereby enabling the development of zone-specific prevention tactics. This methodology paves the way for precision toxicology utilizing modern technologies, including artificial intelligence, to screen segregated zones using an integrated framework composed of the KCCs and cancer hallmarks.

As our spatial-temporal comprehension of cancer deepens with the advent of sophisticated tools and methodologies, there is an opportunity to expand the existing and evolving hallmarks of cancer development and carcinogenesis. This enriches our conceptual model of disease and disease transition starting from pre-disease states. Toxicologists must integrate emerging hallmarks into a comprehensive set of key features, including the existing KCCs, to evaluate routine exposure to potential toxins and mitigate the global health impacts of cancer. Collaboration between KCCs and cancer hallmark communities and the development of a next-generation framework for methods such as NAMs and human exposure -based mechanistic biomarkers are vital for toxicology and cancer research. It is essential to advance cancer prevention strategies, precision environmental health, and align research with the regulatory requirements and global public health needs.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

SS: Writing – review & editing, Writing – original draft, Conceptualization. WB: Writing – review & editing, Conceptualization. AC: Writing – review & editing, Writing – original draft, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2024.1420687/full#supplementary-material

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