Extended Abstracts for the 51st International Symposium of the Princess Takamatsu Cancer Research Fund, 2023

ENVIRONMENTAL IMPACT ON CLONAL EVOLUTION AND CANCER DEVELOPMENT

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Princess Takamatsu Cancer Research Fund

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PRECISION MEDICINE: ENVIRONMENTAL CANCER

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The International Princess Takamatsu Symposium keynote address will be presented in three very brief historical sections: first the status of the field in 1991, then examples of the remarkable progress made by cancer researchers and finally a description of our recent precision medicine studies of lung cancer.

In 1991, it was generally accepted that mutagenesis was a major mechanism of cancer caused by environmental chemicals. An important cellular target was the p53 gene that had been previously discovered by David Lane and L. Crawford[1]. p53 has been found to be the most mutated gene in human cancer. One of the highlights at the 22nd International Princess Takamatsu symposium was the different p53 mutagenesis signatures by aflatoxin B1[2] and other environmental carcinogens. Multiple more complex mutation signatures were initially defined in DNA by Alexandrov and Stratton[3].

This 51st International Princess Takamatsu Symposium further advances our understanding of environmental causes and mechanisms of carcinogenesis.

The keynote address will focus on the major and most costly types of lethal cancers including lung cancer. 25 trillion international dollars during the next 25 years[4], that includes both tobacco smokers and an increasing number of never smokers (Fig. 1 and 2) who were exposed to environmental and passive tobacco carcinogens, air pollutant another causes. The multiomic framework is now defined as precision medicine[5, 6] (Fig. 3).

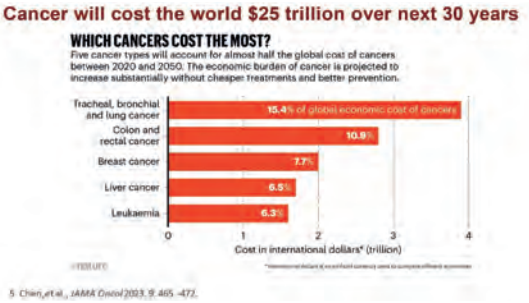


Fig. 1

Fig. 2

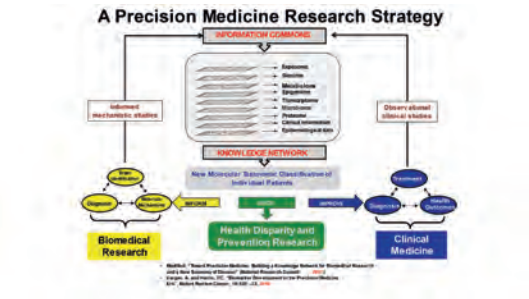
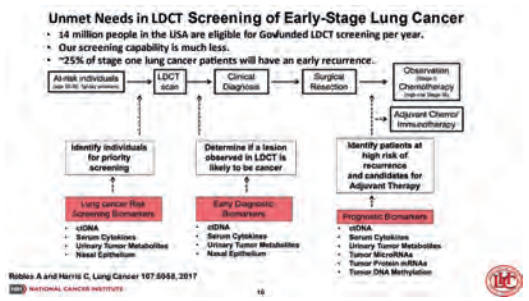
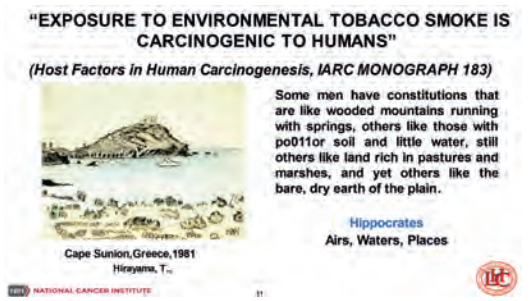


Fig. 3

We will then discuss biomarkers in cancer and liquid biopsy. For example, creatine riboside (CR) is associated with increased risk of multiple types of cancer including lung cancer, brain glioblastoma, breast cancer, prostate cancer and including other types of cancers. Prospective studies have identified individuals at risk of cancer, cancer diagnosis and response to therapy including liquid biopsy of other potential biomarkers in therapeutic pathways and drugs to treat human cancer[7].

The organizers have orchestrated a comprehensive program with themes focused on advancements recently made in identifying cellular and molecular mechanisms of the environmental and occupational cancers. Presentations will update the long-standing themes in carcinogenesis such as tumor promotion, inflammation, DNA addicts, DNA signatures, and the multiomics of precision medicine. Including the microbiome, and the increasing cancer incidence linked to decline of anticancer defenses with increasing age. We have recently been investigating the mechanisms of carcinogenesis and aging-related disease, e.g., Alzheimer's disease, p53 isoforms involvement in cellular senescence[8].





Molecular Epidemiology of Lung Cancer in Never Smokers

Hypothesis:

- Childhood exposure to second-hand smoke and genetic alterations in innate immunity increase lung cancer risk in never smoking adults.

Conclusions:

- Parental secondhand smoke exposure during childhood is associated with dose - dependent increased lung cancer risk among never smokers in two cohorts.
  - Especially among those with an *MBL-2* haplotype with a hyperactive innate immune system
  - Early age of onset of lung cancer

Susan Olivo-Marston

Cancer Epi Bio Prev., 18: 3375 -83, 2009

Collaboration with Jen Jin and Ping Yang, Mayo Clinic

Rationale for LowDose Computed Tomography (LDCT) Screening forNever Smoker Lung Cancer

Increasing Incidence

- >10% of lung cancer cases in the USA; ~25,000 cases
- 20-40% in Asia

Low-Dose Computed Tomography (LDCT)

- Not included in US Government Support LDCT

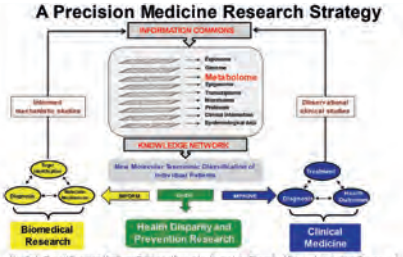
Etiology

- Passive smoking and air pollution(2.5 micron); **Charles Swanton**
- Parental and workplace exposure
- Radon exposures at home and in mines
- Smaller mutational alteration and mutation load compared to smoke

Reviews

- Samet JM; Editorial: J. National Cancer Institute, July 1, 2012
- Alexandrov LB et al., Science 354: 618 -622, 2016

A Precision Medicine Research Strategy



Tumor Metabolism and Metabolome

Hypothesis:

Unbiased metabolomics will discover new biomarkers associated with the risk, diagnosis, prognosis and therapeutic outcome of lung cancer.

Focus on liquid biopsy!

Majda Haznadar

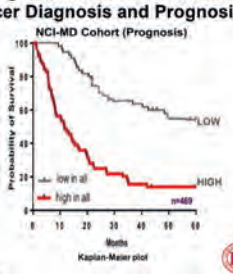
Ewy Mathe

Urinary Metabolomic Profiling Identifies Four Metabolites Associated with Lung Cancer Diagnosis and Prognosis

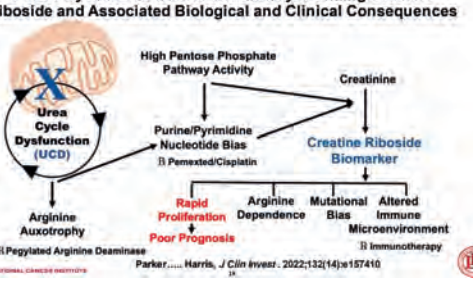
NCI-MD Lung Cancer Case -Control Study[1]

- 469 NSCLC patients
- 536 population controls
- Untargeted Mass Spectrometry
  - RP Chromatography –qTOF MS Analysis
  - Random Forest Analysis for feature identification associated with diagnosis and prognosis
- Four Metabolites
  - Creatine Riboside
  - N-Acetyl Neuraminic Acid
  - Cortisol Sulfate
  - 561.3 (NPC)

Lung Cancer Diagnosis		
Metabolite	All Cases OR (95% CI)	Stage I-II OR (95% CI)
Creatine Riboside	4.88 (3.57 - 7.14)	2.94 (2.07 - 5.24)
N-Acetyl Neuraminic Acid	2.56 (1.93 - 3.59)	1.94 (1.14 - 3.56)
Cortisol Sulfate	2.19 (1.63 - 3.06)	1.72 (1.19 - 3.01)
561.3 NPC	4.28 (3.34 - 6.01)	1.84 (1.18 - 3.11)



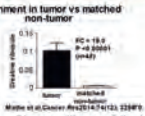
Discovery of the Metabolomic Pathways Forming Creatine Riboside and Associated Biological and Clinical Consequences



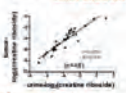
Parker,.... Harris, *J Clin Invest*. 2022;132(14):e157410

Creatine Riboside is Increased in Lung Cancer Tissue Correlation Studies with Liquid Biopsy

Enrichment in tumor vs matched non-tumor

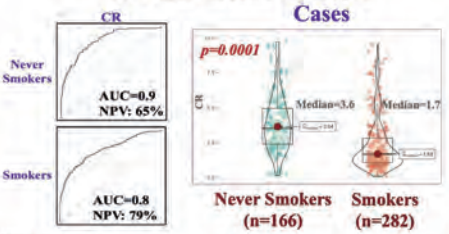


Strong positive correlation between tumor and urine



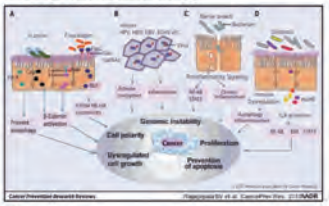
Strong positive correlation between urine and serum or plasma

NCI-MD Cohort Creatine Riboside in Never Smoker vs. Smokers ROC and Violin Blot Analysis

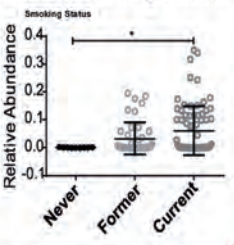


Introduction: Microbiome and Cancer

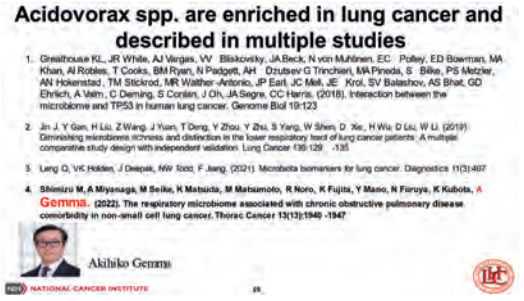
Evidence of bacteria associated with increased inflammation and risk of cancer, such as *H. Pylori* (Gastric cancer), *F. nucleatum* (Colon Cancer), and Dysbiosis (Multiple Cancers, Cancer Therapy).

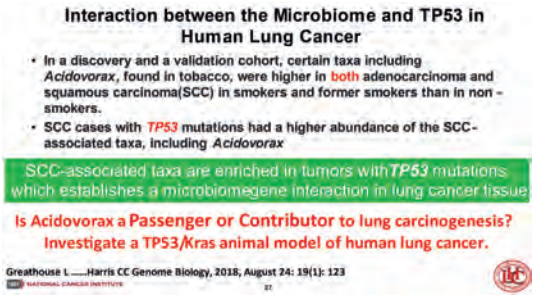


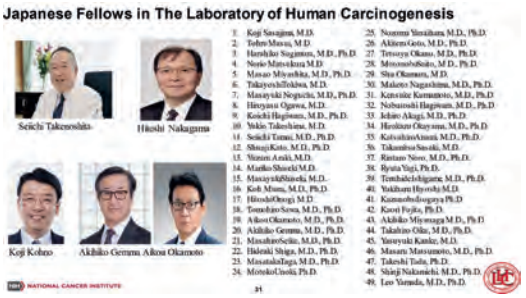
The Acidovorax genus is enriched in lung cancer

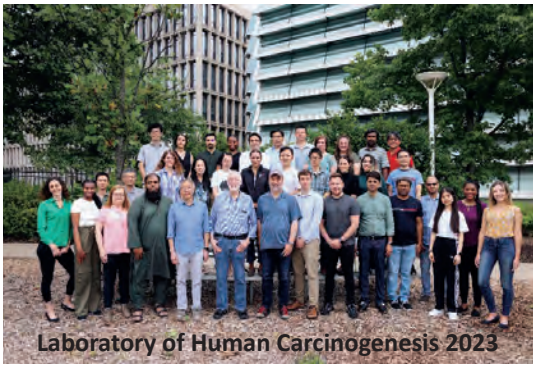


Greathouse et al., *Genome Biol* 19:123, 2018









References

1. D.P. Lane and L.V. Crawford. T antigen is bound to a host protein in SV40-transformed cells, *Nature* 1979. 278: 261-3.
2. C. Hsu, R.A. Metcalf, T. Sun, J.A. Welsh, N.J. Wang and C.C. Harris. Mutational hotspots in the p53 gene in human hepatocellular carcinomas, *Nature* 1991. 350: 427-8.
3. L.B. Alexandrov, Y.S. Ju, K. Haase, P. Van Loo, I. Martincorena, S. Nik-Zainal, et al. Mutational signatures associated with tobacco smoking in human cancer. *Science* 2016. 354: 618-622.
4. S. Chen, Z. Cao, K. Prettner, M. Kuhn, J. Yang, L. Jiao, et al. Estimates and Projections of the Global Economic Cost of 29 Cancers in 204 Countries and Territories From 2020 to 2050. *JAMA Oncol* 2023. 9: 465-472.
5. Modified: Toward Precision Medicine: Building a Knowledge Network for Biomedical Research and a New Taxonomy of Disease. (*National Research Council* 2011).
6. A. Vargas and C.C. Harris. Biomarker Development in the Precision Medicine Era. *Nature Review Cancer* 2016. 18: 525-33.
7. E.A. Mathé, A.D. Patterson, M. Haznadar, S.K. Manna, K.W. Krausz, E.D. Bowman, P.G. Shields, J.R. Idle, P.B. Smith, K. Anami, D.G. Kazandjian, E. Hatzakis, Gonzalez FJ, and Harris CC. Noninvasive urinary metabolomic profiling identifies diagnostic and

prognostic markers in lung cancer. *Cancer Res*. 2014. 74: 3259-70.

8. K. Ungerleider … C.C. Harris. *Neuroscience* 2022. 498:190-202; K Ungerleider … C.C Harris. *Cell Cycle* 2021. 20: 752-764; C. Turnquist … C.C. Harris. *Neuro Oncol* 2019. 21: 474-485; N. von Muhlinen … C.C. Harris. *Oncogene* 2018. 37: 2379-2393; S. Joruiz … C.C Harris. Submitted; J. Beck … C.C. Harris. *Carcinogenesis* 2020 (Review). 41: 1017-1029; and C. Amor … S. W. Lowe. *Nature* 2020. 583: 127-132.

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OCCUPATIONAL URINARY BLADDER CANCERS: INSIGHTS INTO AROMATIC AMINE-INDUCED CARCINOGENESIS

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Occupational bladder cancer is a subtype of bladder cancer resulting from prolonged exposure to carcinogenic agents in the workplace. Aromatic amines, such as o-toluidine (OTD), 2-naphthylamine, 4-aminobiphenyl, and benzidine, are prominent carcinogens linked to this occupational risk. In this symposium, we offer an overview of recent cases of occupational bladder cancer in Japan associated with exposure to OTD and acetoaceto-o-toluidide (AAOT). Furthermore, we share novel insights into the metabolism and carcinogenicity of these aromatic amines, based on our findings from rat and humanized mouse models.

AAOT is utilized as an industrial intermediate in the synthesis of organic pigments. Recently, cases of occupational urinary bladder cancer have been reported among workers in Japanese plants producing AAOT, which utilizes OTD as a raw material. The chronic exposure of these workers to AAOT raised the concerns about its potential link to bladder cancer. In our studies, AAOT induced preneopasitic lesion (simple hyperplasia) on the rat urinary bladder in the short period study [1], and promoted urinary bladder carcinogenesis in a rat two-stage urinary bladder carcinogenesis model (Figure 1) [2]. We also detected that the most abundant urinary metabolite of AAOT was OTD, which was at least one order of magnitude higher than AAOT [1, 2]. These data suggest that AAOT is carcinogenic to the urinary bladder in rats. We also suggest that OTD, which is derived from AAOT metabolism, could be pivotal to AAOT's carcinogenic effects. Mutation analyses reveal that bladder cancers in individuals exposed to either OTD or AAOT have diverse mutations in cancer-related genes, presenting a mutation signature distinct from sporadic bladder cancers. This emphasizes the potential of AAOT, like other carcinogenic aromatic amines, as a human

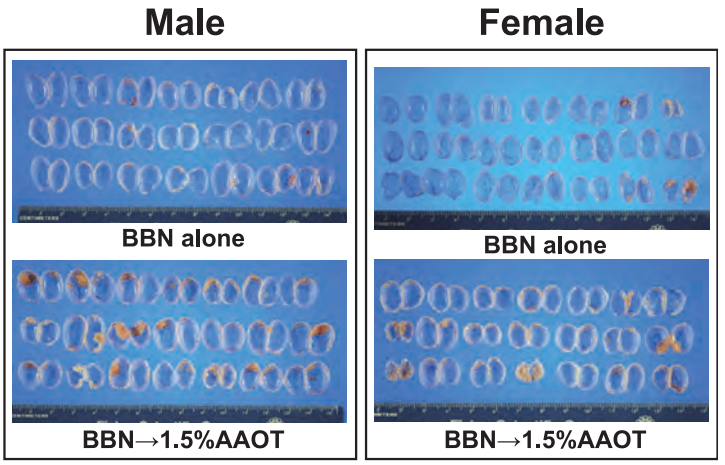

Figure 1 Promoting effects of AAOT on N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN) -induced rat bladder carcinogenesis.

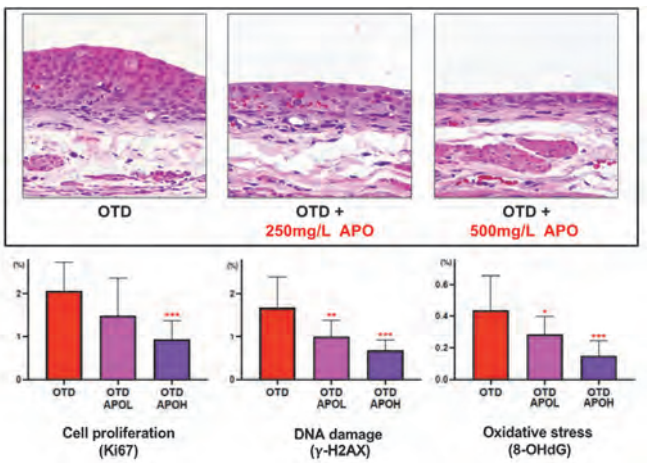

Figure 2 Inhibitory effects of apocynin (APO) on the carcinogenic effects of OTD in the rat bladder epithelium.

bladder carcinogen.

In our study investigating the carcinogenic mechanisms of AAOT and OTD, we identified oxidative DNA adducts, including 8-OHdG, in the urothelium of rats treated with these compounds, using comprehensive DNA adduct analysis. Furthermore, our also found that apocynin, an inhibitor of nicotinamide adenine dinucleotide phosphate oxidase, significantly inhibited cell proliferation, DNA damage, and oxidative stress induced by OTD in a 4-week rat bladder carcinogenicity study (Figure 2) [3]. These findings imply that oxidative stress may play a crucial role in the development of urinary cancer caused by OTD.

In additional studies focusing on human metabolites, OTD was administered to mice with humanized livers, established through human hepatocyte transplantation. This exposure resulted in the induction of CYP3A4 expression in the human liver cells of these mice [4], suggesting that CYP3A4 is an important enzyme involved in the toxicity and carcinogenicity in humans exposed to OTD.

As the list of chemicals associated with workplace exposure continues to grow, identifying chemicals that may trigger occupational bladder cancer becomes increasingly important. Employing experimental models remains an essential approach in determining the causes of occupational cancer and understanding the carcinogenic mechanisms.

References

1. Okuno T, Gi M, Fujioka M, Yukimatu N, Kakehashi A, Takeuchi A, Endo G, Endo Y, Wanibuchi H. Acetoaceto-o-toluidide enhances cellular proliferative activity in the urinary bladder of rats. Toxicol Sci. 2019, 169, 56-64.
2. Yukimatsu N, Gi M, Okuno T, Fujioka M, Suzuki S, Kakehashi A, Yanagiba Y, Suda M, Koda S, Nakatani T, Wanibuchi H. Promotion effects of acetoaceto-o-toluidide on N-butyl-N-(4-hydroxybutyl)nitrosamine-induced bladder carcinogenesis in rats. Arch Toxicol. 2019, 93, 3617-3631.
3. Suzuki S, Gi M, Koyama M, Obikane A, Vachiraarunwong A, Fujioka M, Kakehashi A, Totsuka Y, Wanibuchi H. Evaluation of the mechanisms involved in the development of bladder toxicity following exposure to occupational bladder cancer causative chemicals using DNA adductome analysis. Biomolecules. 2024, 14, 36.
4. Yokota Y, Suzuki S, Gi M, Yanagiba Y, Yoneda N, Fujioka M, Kakehashi A, Koda S, Suemizu H, Wanibuchi H. o-Toluidine metabolism and effects in the urinary bladder of humanized-liver mice. Toxicology. 2023, 488, 153483.

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ENVIRONMENTAL TOXICANTS TARGET PROSTATE STEM-PROGENITOR CELLS TO DRIVE PROSTATE CARCINOGENESIS

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It is long recognized that environmental factors play a major role in cancer etiology, and emerging data suggest that specific toxicants may contribute to an increased cancer risk, including prostate cancer (1). While differentiated prostate epithelial cells can be directly influenced by chemical exposures, properties of stem-progenitor cells make them compelling targets of tumorigenesis. Stem cells are fundamental components of biological organization, responsible for development and maintenance of tissues and organ systems (2). Embryonic stem cells are pluripotent cells with a robust proliferative capacity and ability to differentiate into the three embryonic germ cell layers. Once tissues and organs are formed following morphogenesis, adult tissue-specific stem cells maintain homeostasis within that structure, providing cells for natural tissue turn over and regeneration as well as response to injury. The highly plastic state of stem and daughter progenitor cells during development and tissue maintenance permits the needed flexibility for proper tissue formation and repair. Regrettably, this plasticity also provides an opportunity for aberrant cellular reprogramming due to inappropriate signaling from chemical exposures that can lead to persistent life-long effects and tissue perturbation. Further to this point, the cancer stem cell hypothesis identifies normal tissue stem cells and their immediate progenitors as putative targets for cell transformation and tumor initiation (3, 4).

While stem and progenitor cells in all systems are tightly regulated by their microenvironment or stem cell niche, hormonally sensitive tissues appear to have an additional layer of hormonal regulation of the stem and progenitor cells. Our research has shown that prostate stem and progenitor cells (SPCs) express nuclear receptors that can tightly regulate stem cell self-renewal and progenitor cell lineage commitment and

differentiation. Although negative for androgen receptor (AR), the human prostate SPC population expresses estrogen receptors (ERα, ERβ, GPER), retinoid receptors (RARs, RXRs), vitamin D receptor (VDR), peroxisome proliferator receptors (PPARs), Aryl Hydrocarbon receptor (ArR) among others (5, 6). Early evidence demonstrates that when activated by their cognate ligands, these receptors mediate diverse effects including stem cell self-renewal, progenitor cell amplification and differentiated lineage commitment. Unfortunately, exposures to endocrine disrupting chemicals and other environmental compounds have the potential to disrupt prostate homeostasis through these same pathways. As such, research in my laboratory focuses on these long-lived cells as direct environmental toxicant targets. Our findings have determined that several compounds, including Bisphenol A (BPA), Per-and polyfluorylalkyl substances (PFAS), and inorganic arsenic, reprogram normal human prostate SPC populations, leading to increased cancer susceptibility. Of particular note, the observed alterations in SPC homeostasis leading to transformation are mediated through chemical-specific mechanisms.

Direct Effects of Bisphenol A on Human Prostate Stem and Progenitor Cells that Increase Carcinogenic Susceptibility in Human Prostate Epithelium

To directly assess the actions of endogenous and exogenous factors on prostate SPCs and to evaluate the relevance of these findings to the human prostate gland, we derived *in vitro* and *in vivo* systems utilizing primary cells cultured from prostates of young, disease-free organ donors. Adult prostate stem cells were enriched by FACS or 3-D matrigel culture to form prostaspheres (PS) (7). Exposure of primary human prostate epithelial cell cultures to estradiol-17β results in an in stem-like cell numbers using the side-population FACS assay and increased PS numbers and size indicative of enhanced stem cell self-renewal and progenitor cell proliferation, respectively (8). Similarly, exposure to BPA, a ubiquitous estrogenic-like chemical found in 1000s of consumer products, enhanced SPC growth at environmentally relevant levels and augmented expression of stemness genes (NANOG, TBX3).

To understand the molecular underpinnings of BPA effects, the transcriptome and epigenome of SPCs were interrogated in spheroids grown in the presence of vehicle, estradiol or BPA (9). Using an Affymetrix human genome microarray, a unique block of 26 genes was consistently down regulated across all donors and treatment groups of which 15 were identified as small noncoding nucleolar RNA (SNORDs). While the SNORDS were not silenced by DNA methylation modifications, they were repressed by specific histone modifications at H3K4, H3K9 and H3K27 (9). Together, these findings show that estrogenic chemicals modify the epigenome of normal adult prostate SPCs. Since epigenetic mechanisms are crucial in maintaining stem cell pluripotency and controlling differentiation

into progenitor cells and epithelial cell lineages (10), alterations in epigenetic marks may set the stage for aberrant differentiation programs within the prostate epithelium which may, in turn, contribute to increased human prostate cancer risk.

We next demonstrated increased carcinogenic risk in prostate epithelium following progenitor cell exposure to BPA through creation of an *in vivo* model using the SPCs isolated from normal human prostates. Prostaspheres of SPCs were dispersed, combined with inductive rat urogenital sinus mesenchyme and grafted under the renal capsule of adult male nude mice (8), forming normal prostate-like structures with differentiated human prostate epithelium that expressed prostate specific antigen (PSA). Once mature tissues formed, host mice were treated with elevated estradiol (E) and testosterone (T) supplementation for 2-4 months to drive carcinogenesis. Pathologic lesions were observed in the human prostate epithelium over time, progressing from hyperplasia, squamous metaplasia and high-grade PIN to adenocarcinoma at a low incidence. To test whether BPA could influence this process, host mice were orally exposed to low doses of BPA for two weeks following engraftment, followed by T+E exposure for 4 months. The incidence of malignancy in the human prostate epithelium significantly increased from 13% in oil-exposed controls given T+E to 33-36% in tissues exposed *in vivo* to BPA followed T+E. This further increased to 45% cancerous lesions when the SPCs were exposed to BPA *in vitro* followed by 2 weeks of *in vivo* exposure. Together, these results demonstrate that exposures to low doses of BPA increase the susceptibility of the human prostate epithelium to estrogen-driven carcinogenesis and that prostate SPCs are direct targets that propel this increased cancer risk.

Inorganic arsenic transforms human prostate progenitor cells through p62-KEAP-NRF2

Inorganic arsenic (iAs) is a ubiquitously distributed environmental and industrial toxicant, classified as a class I carcinogen by the International Agency for Research on Cancer (11). More than 137 million people in over 70 countries are exposed to iAs at levels of greater than 10 ppb, the U.S. Environmental Protection Agency drinking water standard (12). Epidemiologic studies and experimental evidence link chronic iAs exposure with increased risk of certain cancers (11, 13) and while not conclusive, emerging epidemiological data suggests a direct causal relationship between iAs exposure and prostate cancer incidence and mortality (14). As some studies using human prostate cell lines identified prostate SPCs as arsenic targets for transformation (15), we investigated whether prostate SPCs from disease-free organ donors were direct iAs targets and sought to identify the underlying mechanisms of transformation.

Using the model systems described above, we found that exposure to 1 μM iAs (~75 ppb) significantly increased stem cell numbers, inhibited cell differentiation in organoid cultures

and induced progenitor cell transformation as assessed by soft-agar colony formation. Transcriptomic analysis found that brief exposure of prostaspheres (PS) to 1 μM iAs augmented prostate carcinogenic pathways (kRAS, PTEN, p53, MEK), including activation of the KEAP1-NRF2 pathway. NRF2 was deemed essential for iAs-induced transformation of progenitor cells which was suppressed by NRF2 knockdown. Detailed studies determined that this was mediated by a non-canonical p-p62 dependent pathway, elevated during autophagosome formation which allowed for KEAP1-NRF2 dissociation and translocation of NRF2 to the nucleus. Further, we established that autophagy-flux blockade underpinned the p-p62 accumulation. Mechanistically, iAs suppressed V-ATPase subunit VMA5 expression, impairing lysosome acidification and inhibiting autophagic protein degradation including p62. *In vivo*, chronic iAs exposure activated NRF2 in both epithelial and stroma cells of chimeric human prostate grafts and induced premalignant events. In summary, we outlined a novel mechanism involving NRF2 dysregulation by which iAs interferes with homeostasis of prostate SPCs and predisposes to tumorigenesis.

Per- and Polyfluoroalkyl Substances Target and Alter Human Prostate Stem-Progenitor Cells

Per- and polyfluoroalkyl substances (PFAS) are synthetic chemicals utilized in various industrial settings, and include products such as flame retardants, artificial film-forming foams, cosmetics, non-stick cookware among others. Epidemiological studies suggest a link between increased blood PFAS levels and prostate cancer incidence and our in vitro and in vivo studies with benign and cancerous prostate cell lines confirmed the enhancement of cell and tumor growth when exposed to elevated levels of several PFAS compounds (16). PFAS chemicals are known to bind to PPARs in other organ systems, and since prostate stem-progenitor cells express PPARs, we asked whether they are direct PFAS targets that may be involved in PFAS-driven prostate cancer incidence and mortality. Exposure of normal human prostate SPCs to 10 nM PFOA or PFOS for four weeks using serial passage of prostasphere cultures, markedly increased spheroid numbers and size indicative of elevated stem cell self-renewal and progenitor cell proliferation (17). Transcriptome analysis using single-cell RNA sequencing (scRNA-seq) confirmed enrichment of stem cells and early-stage luminal cells, decreased basal progenitors and the emergence of a new cell cluster of aberrantly differentiated luminal progenitor cells upon PFOS/PFOA exposure indicating altered lineage commitment. Pathway analysis found enrichment of cancer-associated signaling pathways and enhanced glycolosis pathways. Metabolomic analysis of PFAS-exposed prostaspheres revealed increased glycolytic pathways including the Warburg effect as well as strongly enrichment of serine and glycine metabolism which may promote a pre-malignant SPC fate. Together, these findings identify human prostate SPCs as direct PFAS targets with resultant reprogrammed transcriptomes and an oncogenic metabolome that

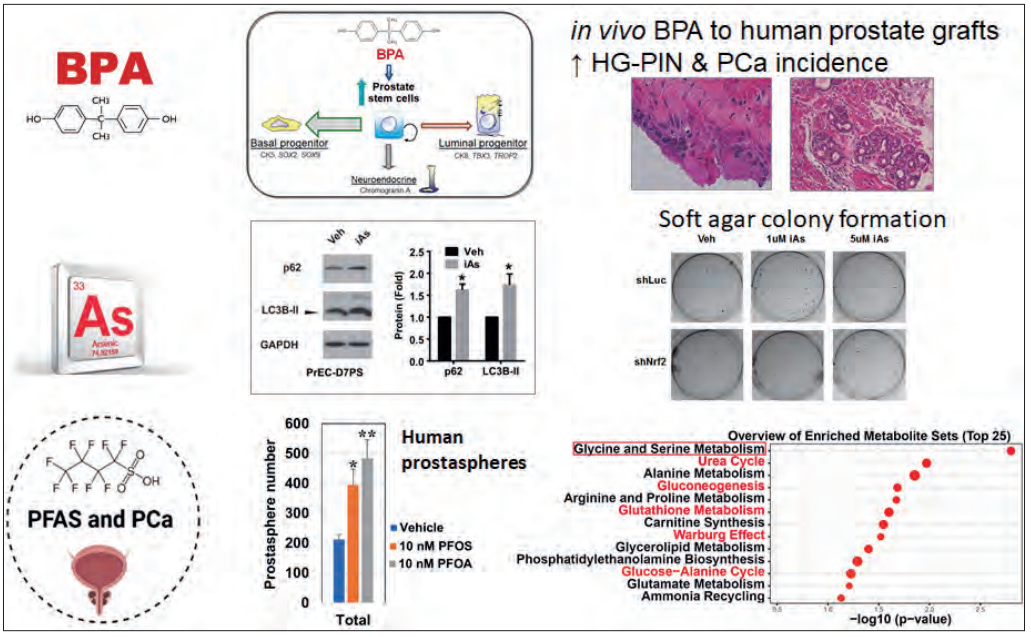

Figure 1 Environmental toxicants modify prostate SPC homeostasis that underpins transformation in a chemical-specific manner.

may contribute to an elevated prostate cancer risk with chronic exposures.

In summary, our findings document that prostate SPC populations are direct targets for many environmental toxicants that reprogram and transform the cells through chemical-specific mechanisms (Fig 1). Due to the long-lived nature of prostate stem cells, these processes can underpin increased prostate carcinogenic potential over extended time periods.

References

1. L X, GS P. Environmental Factors in Cancer Risk. In: D H, Z L, VLW G, eds. Nutritional Oncology: Nutrition in Cancer Prevention, Treatment and Survivorship. Vol First Edition: CRC Press; 2022:459-474.
2. Weissman IL. Stem cells: units of development, units of regeneration, and units in evolution. *Cell*. 2000;100(1):157-168.
3. Wicha MS. Targeting breast cancer stem cells. *Breast*. 2009;18 Suppl 3:S56-58.
4. Batlle E, Clevers H. Cancer stem cells revisited. *Nature medicine*. 2017;23(10):1124-1134.
5. Prins GS, Hu W. Prostate Stem Cells, Hormones and Development. In: SD C, ed. Stem Cells and Prostate Cancer. Vol VII: Springer LLC; 2013:1-20.
6. Hu WY, Shi GB, Hu DP, Nelles JL, Prins GS. Actions of estrogens and endocrine disrupting chemicals on human prostate stem/progenitor cells and prostate cancer risk.

Molecular and cellular endocrinology. 2012;354(1-2):63-73.

7. Hu WY, Shi GB, Lam HM, Hu DP, Ho SM, Madueke IC, Kajdacsy-Balla A, Prins GS. Estrogen-initiated transformation of prostate epithelium derived from normal human prostate stem-progenitor cells. *Endocrinology*. 2011;152(6):2150-2163.
8. Prins GS, Hu WY, Shi GB, Hu DP, Majumdar S, Li G, Huang K, Nelles JL, Ho SM, Walker CL, Kajdacsy-Balla A, van Breemen RB. Bisphenol A promotes human prostate stem-progenitor cell self-renewal and increases in vivo carcinogenesis in human prostate epithelium. *Endocrinology*. 2014;155(3):805-817.
9. Ho SM, Cheong A, Lam HM, Hu WY, Shi GB, Zhu X, Chen J, Zhang X, Medvedovic M, Leung YK, Prins GS. Exposure of Human Prostaspheres to Bisphenol A Epigenetically Regulates SNORD Family Noncoding RNAs via Histone Modification. *Endocrinology*. 2015;156(11):3984-3995.
10. Mikkelsen T, Ku M, Jaffe D, Issac B, Lieberman E, Giannoukos G, Alvarez P, Brockman W, Kim T, Koche R, Lee W, Mendenhall E, O'Donovan A, Presser A, Russ C, Xie X, Meissner A, Wernig M, Jaenisch R, Nusbaum C, Lander E, Bernstein B. Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. *Nature*. 2007;448:553-560.
11. IARC. A review of human carcinogens: Part C: metals, arsenic, dust and fibres. *IARC Monogram*. 2009:453-454.
12. Carlin DJ, Naujokas MF, Bradham KD, Cowden J, Heacock M, Henry HF, Lee JS, Thomas DJ, Thompson C, Tokar EJ, Waalkes MP, Birnbaum LS, Suk WA. Arsenic and Environmental Health: State of the Science and Future Research Opportunities. *Environmental health perspectives*. 2016;124(7):890-899.
13. Tokar EJ, Benbrahim-Tallaa L, Ward JM, Lunn R, Sams RL, Waalkes MP. Cancer in experimental animals exposed to arsenic and arsenic compounds. *Critical reviews in toxicology*. 2010;40(10):912-927.
14. Yang Y, McDonald AC, Wang X, Pan Y, Wang M. Arsenic exposures and prostate cancer risk: A multilevel meta-analysis. *Journal of trace elements in medicine and biology : organ of the Society for Minerals and Trace Elements (GMS)*. 2022;72:126992.
15. Tokar EJ, Diwan BA, Waalkes MP. Arsenic exposure transforms human epithelial stem/progenitor cells into a cancer stem-like phenotype. *Enivon Health Perspect*. 2010;118(1):108-115.
16. Imir OB, Kaminsky AZ, Zuo QY, Liu YJ, Singh R, Spinella MJ, Irudayaraj J, Hu WY, Prins GS, Madak Erdogan Z. Per- and Polyfluoroalkyl Substance Exposure Combined with High-Fat Diet Supports Prostate Cancer Progression. *Nutrients*. 2021;13(11).
17. Hu WY, Lu R, Hu DP, Imir OB, Zuo Q, Moline D, Afradiasbagharani P, Liu L, Lowe S, Birch L, Griend DJV, Madak-Erdogan Z, Prins GS. Per- and polyfluoroalkyl substances target and alter human prostate stem-progenitor cells. *Biochemical pharmacology*. 2022;197:114902.

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MECHANISM OF ACTION AND AN INFLAMMATORY AXIS FOR AIR POLLUTION-INDUCED NON-SMALL CELL LUNG CANCER

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Lung cancer in never-smokers is distinct from smoking-associated lung cancer in that it has a low mutational burden and over half of patients have tumours with an *EGFR* mutation. Proposed risk factors have included germline susceptibility loci, radon gas, second-hand smoke, and environmental air pollution.

We have hypothesised that environmental carcinogens like environmental pollution or second-hand smoke might cause lung cancer by promoting the expansion of pre-existing mutant clones.

Work from TRACERx has demonstrated that lung cancer in never-smokers is associated with a low mutational burden that has very few mutations. Those mutations tend to be in an ageing context or APOBEC context. The Sherlock study has demonstrated that there is no carcinogenic mutational signature in 97% of Sherlock study never-smoking lung cancers. Approximately 70 years ago, Doll and Hill were amongst the first to propose a link between atmospheric air pollution from exhaust fumes and lung cancer, but in their seminal paper in 1950 proved the association of tobacco with lung cancer. Over the course of the last four decades, there've been at least six meta-analyses demonstrating an association of air pollution with both lung cancer in smokers and lung cancer in never-smokers. The relative risks appear to be greater in lung adenocarcinoma. We hypothesised that if environmental air pollution is causing lung cancer in never-smokers it would have to fulfil four criteria. It would have to explain the geographic distribution of the disease, we would have to prove that pollution directly causes lung cancer, we would have to explain how pollution could

cause cancer without directly causing DNA mutations and explain why lung cancer in never-smokers is more common in females. We reasoned that the classic mutational model might not apply to environmental air pollution-associated lung cancer or lung cancer caused directly by air pollution for the reason that there is no clear environmental carcinogenic signature in these tumours. Instead, a potential model similar to the tumour promotion model as first proposed by Berenblum in 1947 might be operating. Berenblum, and latterly Balmain, proposed that tumours are promoted from cells with pre-existing mutations. We wondered whether air pollution might act as a tumour promoter rather than a mutagenic initiator.

Firstly, we conducted a pan-cancer analysis of cancer incidence in PM2.5s in UK biobank participants. In over 407,000 participants we demonstrated an increased risk of lung cancer controlled for multiple testing of approximately 1.08 for each $\mu g/m^3$ increment of PM2.5. We went on to find that PM2.5s are associated with *EGFR* mutant lung cancer incidence in England, Taiwan, and South Korea. These data are consistent with a meta-analysis for adenocarcinoma diagnoses per 5 $\mu g/m^3$ rise in PM2.5s of approximately 1.55. Next, we set up three GEMM models of *EGFR* or *KRAS*-mutant lung cancer and instilled intratracheal PM2.5s every two days for three weeks in these mice after oncogene induction. In all three models, we found an increased incidence of cancer lesions or adenoma lesions in each of the mouse models in a dose-dependent manner. We could find no evidence of clear mutagenic signatures in these tumours and no significant increase in the number of mutations when these air pollution-associated tumours were subjected to whole genome sequencing compared to saline control. Therefore, it did appear that Berenblum's model might be operating in these tumours. Next, we demonstrated that there was no significant rise in air pollution associated lung cancers in a RAG2 IL-2 receptor gamma null mouse model deficient in T and B cells as well as NK cells. We saw no increase at 50 μg of PM 2.5 exposure in the number of lesions per millimetre squared in mouse lungs suggesting that a competent immune system is required for PM-mediated tumorigenesis. Next, we subjected mouse epithelial cells to transcriptional analysis and found evidence of an AT2 cell state and a macrophage recruitment state. Subsequent experiments demonstrated that a progenitor cell state was initiated following air pollution exposure of mouse epithelial cells but only in the presence of an *EGFR* activating mutation. This appeared to be secondary to macrophage infiltrates in epithelial tissue following PM 2.5 exposure. We found evidence of increased macrophage infiltrates in both mouse and human lungs exposed to air pollution. Analysis of published data from Christopher Carlston and colleagues demonstrated interleukin-1 beta (IL-1β) upregulation in both mouse and human lung tissue after acute PM2.5 exposure. These data were pertinent given the results of the Cantos trial which has shown a dose-

dependent reduction in new lung cancer primaries following anti-IL-1β therapy. Treatment of mice with an anti-IL-1β antibody appeared to attenuate new tumour formation in the presence of air pollution and anti- IL-1β antibody. We found evidence that one of the initiating cells is the AT2 cell. Therefore, the AT2 cell appears to be a PM2.5 vulnerable tumour cell-of-origin. Follow up experiments demonstrated that pollution exposed macrophages elevate progenitor capacity of *EGFR* mutant AT2 cells exposed to room air.

Finally, in order to demonstrate that the Berenblum model might be correct, we had to demonstrate whether we had to assess whether there was evidence of mutant clones in normal tissue. Analysis of normal tissues using digital PCR with SAGA technologies or TwinStrand Duplexseq analysis demonstrated *EGFR* activating mutations in 15% of normal lung tissues and *KRAS* activating mutations in 53% of normal lung samples. These mutations appeared to expand in pollution exposed lung and smoking exposed lung respectively. These mutations appeared to occur due to the ageing process with evidence of an increased number of activating mutations found in normal lung tissue with age in never smokers. In summary, our data suggest that air pollution acts as an inflammatory mediator driving the expansion of pre-existing oncogenic mutations in normal tissue in vulnerable progenitor cells such as the AT2 cell. These data are consistent with animal model systems published by both Balmain and Berenblum suggesting a route into molecular cancer prevention by blocking inflammatory pathways that might sustain or initiate the expansion of pre-existing mutant clones in progenitor cells.

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HIGH-RESOLUTION MASS SPECTROMETRY-BASED APPROACHES TO IMPROVE THE CHARACTERIZATION OF CHEMICAL CARCINOGENESIS

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Characterizing chemical exposures and their health effects in humans, remains a significant challenge. This is due in part to the difficulties related to capturing the complexity of exposures and their interaction with biological systems, but also to the fact that markers that can help disentangle this complexity, are often at trace levels. Improved tools providing greater analytical sensitivity, combined with comprehensive screening capabilities, are needed to move this field of research forward. Our laboratory is focusing on developing state-of-the-art high-resolution mass spectrometry-based methods to characterize the exposome and determine how it may interact with cellular targets playing a key role in carcinogenesis. These methods are based on the use of high-field orbital trap instrumentation (Thermo Scientific Orbitrap Lumos) which is very well suited for the analysis of challenging low-abundance, high-complexity samples. We are developing approaches based on neutral loss screening techniques paired with relative quantitation strategies to perform metabolic profiling focused on the characterization of reactive chemicals in biological fluids. In parallel, we are working on establishing a DNA adductomic approach based on a data dependent-constant neutral loss-MS^3 (DDA-CNL/MS^3) methodology as a comprehensive screening method to characterize all covalent DNA modifications (DNA adducts), induced by various exposures.[1] We are now applying this approach for a more comprehensive characterization of the DNA damage resulting from genotoxic exposures, such as tobacco smoke, the characterization of unknown genotoxins produced by the gut microbiome and known to be linked to increased risk of colon cancer, and to the development of markers to support stratification of patients undergoing chemotherapy based on alkylating agents. An overview of these three lines of research is presented.

In the case of tobacco smoke, the DNA damage resulting from the interaction of the tobacco specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is analyzed in the presence of inflammation, to investigate the driver adducts that are enhanced by the inflammatory process and that may be responsible for the initiation of carcinogenesis. Lung DNA samples from an animal model in which animals are exposed to NNK and the combination of NNK plus the inflammation-inducing agent Lipopolysaccharide (LPS) are analyzed and compared to tissue samples from animals exposed to LPS only and controls. This model is used because animals develop lung tumors after several weeks and the effect is increased in severity and number of tumors when animals are co-exposed with NNK and LPS. Therefore, this model allows to monitor and follow the development of the DNA damage from its initial formation to the actual development of tumors, and allows to fully characterize the overall carcinogenic process.[2] The use of our DNA adductomic approach allowed to detect and measure a large number of DNA adducts generated by NNK, including expected monoadducts, protein and DNA cross links and it also allowed to discover some modifications previously unknown, namely DNA-RNA crosslinks.[3] The method is now used to analyze the development of the profile of adducts over time in the same animal model, by analyzing lung DNA samples from animals exposed to NNK or LPS and NNK at 1, 5 and 15 weeks after initial exposure. Also, in this case the results are compared with samples collected from animals only treated with LPS or with the vehicle as controls.

In the case of using our DNA adductomic approach to discover new genotoxins produced by the gut microbiome, we investigated the DNA damaging effects of the genotoxin colibactin, produced by the *E.coli* carrying the pks+ island and known to be associated with colorectal cancer risk. This genotoxin has never been characterized before and the elucidation of the modification it results in when binding DNA allowed us to understand the structural features of the original molecule.[4] Structural elucidation was possible by taking advantage of the various levels of fragmentations at high resolution that our method features. The identified adduct was then analyzed and found in tissue from animals exposed to *E.coli* pks+ and was further investigated in samples obtained from human colonoscopies. The human samples included in this work were collected from a biorepository created at the University of Minnesota, and approved by the University IRB. which includes Cystic Fibrosis patients. These patients are at higher risk of developing colon cancer and their disease disrupts the permeability of their gut mucosa. Therefore, samples from these patients were expected to potentially be more susceptible to the presence of the colibactin-derived DNA adduct. The DNA adducts analysis of 85 Cystic Fibrosis samples was compared to that of 59 controls after assessing the infection to E.coli pks+ using qPCR. Around 30% of participants were positive to the infection, however only 2 individuals, among the Cystic Fibrosis group, were

found to be positive to the presence of the colibactin-derived DNA adducts. Interestingly these two patients had several characteristics in common: they were both obtained from lung transplant patients who were not taking any medication for Cystic Fibrosis and who were diagnosed with colon cancer or a precancerous lesion during the colonoscopy. Furthermore, the E.Coli pks+ infection did not seem to be enough for the detection of the adducts and other parameters seemed to be necessary for the adduct to be detected. These preliminary findings need to be further confirmed on a larger sample size. However, this is the first report of the presence of this adduct in human samples obtained from normal tissue collected during screening colonoscopies.

Finally, our method has been used to characterize the DNA damage deriving from DNA alkylating drugs used in chemotherapy, to investigate the potential use of the corresponding DNA adducts as markers to stratify patients and discriminate those who may experience toxicity or less effective outcomes from those benefiting from the treatment. Cyclophosphamide-derived DNA adducts were characterized *in vitro* by exposing calf thymus DNA to the drug in the presence of S9 metabolism and comparing the DNA adduct profile present in the exposed DNA only and not in the control DNA. A list of 40 adducts was compiled. These DNA modifications were then investigated in peripheral blood DNA of patients undergoing cyclophosphamide treatment as part of the preparative regimen for bone marrow transplant. Twenty cyclophosphamide-derived DNA adducts were detected in patients' DNA. These findings set the stage for the investigation of the relationship between each of these DNA modifications and therapy outcome, to indentify any DNA adduct associated with severe toxicity or lower response to the treatment. These investigations are aiming at developing markers to be used to predict response to therapy and adapt the regimen type and dosage in a more precise and personalized fashion.[5]

The examples presented, show the power of the MS-based methods that we are developing aimed at generating innovative comprehensive tools for the characterization of exposures in humans and for the elucidation of their effects on DNA. These approaches have important implications for the development of new genotoxicity testing methods, the development of new biomonitoring tools, and the development and support of improved cancer prevention and treatment strategies.

References

1. Stornetta A, Villalta PW, Hecht SS, Sturla SJ, Balbo S. Screening for DNA Alkylation Mono and Cross-Linked Adducts with a Comprehensive LC-MS(3) Adductomic Approach. Anal Chem. 2015 Dec 1;87(23):11706-13. doi: 10.1021/acs.analchem.5b02759. Epub 2015 Nov 10. PMID: 26509677; PMCID: PMC5126974.

2. Melkamu T, Qian X, Upadhyaya P, O'Sullivan MG, Kassie F. Lipopolysaccharide enhances mouse lung tumorigenesis: a model for inflammation-driven lung cancer. Vet Pathol. 2013 Sep;50(5):895-902. doi: 10.1177/0300985813476061. Epub 2013 Feb 4. PMID: 23381924; PMCID: PMC4667800.
3. Dator RP, Murray KJ, Luedtke MW, Jacobs FC, Kassie F, Nguyen HD, Villalta PW, Balbo S. Identification of Formaldehyde-Induced DNA-RNA Cross-Links in the A/J Mouse Lung Tumorigenesis Model. Chem Res Toxicol. 2022 Nov 21;35(11):2025-2036. doi: 10.1021/acs.chemrestox.2c00206. Epub 2022 Nov 10. PMID: 36356054; PMCID: PMC10336729.
4. Wilson MR, Jiang Y, Villalta PW, Stornetta A, Boudreau PD, Carrá A, Brennan CA, Chun E, Ngo L, Samson LD, Engelward BP, Garrett WS, Balbo S, Balskus EP. The human gut bacterial genotoxin colibactin alkylates DNA. Science. 2019 Feb 15;363(6428):eaar7785. doi: 10.1126/science.aar7785. PMID: 30765538; PMCID: PMC6407708.
5. Guidolin V, Jacobs FC, MacMillan ML, Villalta PW, Balbo S. Liquid Chromatography-Mass Spectrometry Screening of Cyclophosphamide DNA Damage In Vitro and in Patients Undergoing Chemotherapy Treatment. Chem Res Toxicol. 2023 Aug 21;36(8):1278-1289. doi: 10.1021/acs.chemrestox.3c00008. Epub 2023 Jul 25. PMID: 37490747.

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DNA ADDUCTOMICS IN HUMAN TISSUES

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"Adduct" is a synthetic term of "addition" and "product", and "adductomics" is meant for an approach of comprehensive capture of numerous adducts generated both endogenously and exogenously in various biological systems. In the context of environmental and/or chemical carcinogenesis, DNA adducts formation, with deficient or insufficient repair of them, is a pivotal step toward carcinogenic mutagenesis (Fig 1). From the standpoint DNA lesions, DNA adducts including base modifications by endogenous enzymes, theoretically, are the most proximal or ultimate indicators of exposures, a consequence of "exposomics".

DNA adducts in tissues: Since successful formation of artificial cancer in rabbits [1], animal models and *in vitro* models of chemical carcinogenesis have provided theoretical bases for how cancer arises, that is, the basis of cancer science. They lead to the concept of cancer hallmarks and precision therapy of cancers.

Fig.1 Adducts formation in the pathway from exposure to mutations

In animal models, most often overdosed by chemicals, detections of DNA adducts and investigation of subsequent mutation spectrum/signature further established the main theorem of carcinogenesis down to the details. DNA adducts in these animal models have been extensively reviewed focusing several different pathways [2, 3], such as alkyl adducts and polyaromatic hydrocarbon adducts, according to different target organs.

Extrapolation to human beings: As we know, understandings on human carcinogenesis based on these basic and experimental findings are dramatically crystalized in recent achievements, comprehensive cataloguing of somatic DNA changes of various human cancers and reasoning these somatic changes as a reflection of carcinogenesis process including environmental exposures and life styles. Large scale acquisition of DNA mutations based on massive parallel sequencing technology have also paved the way to current targeted therapy of individual cancers according to individual somatic changes. Recently clonal and subclonal mutations in apparently normal human tissues became detectable and a possibility to use them as indicators of risk in individuals is argued.

On the other hand, an adductomics approach, detection and interpretation of DNA adducts in daily medical practice, though we used to expect it help identifying specific exposure and specific high-risk populations or individuals progresses more slowly.

The challenges in detection, identification, and quantitation of DNA adducts in human tissues were and still are in methodology and accessibility. ^{32}P post-labelling method was widely applied to detect DNA adducts in various settings including human tissues, but specific identification of DNA adducts, supposed to exist as several different modified bases, was not available. For the last two decades combination of double mass spectrometry and liquid chromatography enabled us to identify dozens of specific DNA adducts at single experiment [4]. Now we became able to compare the tissue originated from animals exposed to experimental carcinogens and from human tissues having cancers and resected by surgery or autopsy. The findings would provide us additional and applicable information on straightforward interpretations or misinterpretations of environmental or chemical carcinogenesis of human and other animals.

Theoretically, when we have chemical standards of DNA adducts (isotopic ones) synthesized we would be able to identify and estimate as many as the numbers of these standard compounds. We called it "adductome map" [4] (Fig 2). The first adductome map was drawn based on just two autopsy lung tissues from a smoker and a never-smoker. Circle size in Cartesian plane represent quantities of DNA adducts or modification calculated from the LC-MSMS. Annotations of the circles are far from complete, and detectability and semi-quantitation are the greatest challenges, too. Some adducts, nevertheless, are repeatedly

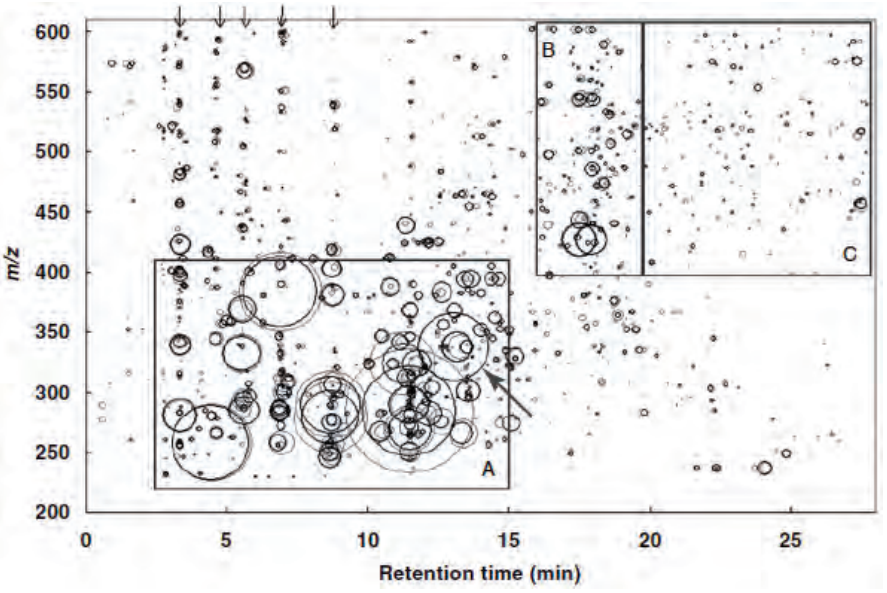

Fig 2 Adductome map from two lungs, a smoker (dark circle) and a never-smoker (light circle). From the reference 4. Only some of the circles have annotations.

detected in human tissues.

Lipid peroxidation derived DNA adducts from different geographical populations: Lipid peroxidation derived DNA adducts are detected at considerable levels in various organs of human tissues [5]. Our currently used protocol to identify and quantitate is relatively stable and established. Then adductomics approach focusing these 7 DNA adducts was applied to gastric mucosa from two populations. 12 and 10 cases of gastric mucosa were recruited from Lujiang Hospital (Lujiang County, Anhui Province, China, the 5th highest county of all the counties in China, 2010) and Hamamatsu University Hospital (Hamamatsu, Japan). 7 lipid peroxidation derived DNA adducts were identified semi-quantitatively. Seven lipid peroxidation-related DNA adducts [1,N6-etheno-2'-deoxyadenosine(εdA), butanone-etheno-2'-deoxycytidine (BεdC), butanone-etheno-2'-deoxy-5-methylcytidine, butanone-etheno-2'-deoxyadenosine (BεdA), heptanone-etheno-2'-deoxycytidine, heptanone-etheno-2'-deoxyadenosine (HεdA) and heptanone-etheno- 2'-deoxyguanosine] were identified in a total of 22 gastric mucosa samples. The levels of these adducts ranged from 0 to 30,000 per 10^9 bases. Although the presence of Helicobacter pylori DNA in the mucosa was not related to these adducts level, the levels of BεdC, BεdA and HεdA were higher in the Japanese gastric mucosa samples. The profiles of these 7 adduct levels among the 21 cases were capable of discriminating between the possible origins (China or Japan) of the gastric mucosa samples. One outlier case was revealed to be EBV associated cases, with massive lymphoid

cell infiltration. From this experience, we think we will be able to suspect of geographic origin of cancers by revealing adductomics profile [6].

Geospatial distribution of DNA adducts in human stomach: Gastric cancer arises more frequently in the lesser curvature than greater curvature, thus geospatial distribution of DNA adducts, presumable factors for carcinogenesis may influence this preferred region of cancer occurrence in the human stomach. We did take DNAs from up to 27 sites per case from individual resected stomach for gastric cancer. We compared the quantities of C5-methyl-2′-deoxycytidine, 2′-deoxyinosine, C5- hydroxymethyl-2′-deoxycytidine, N6-methyl-2′-deoxyadenosine, εdA, N6-hydroxymethyl- 2′-deoxyadenosine, and C8-oxo-2′-deoxyguanosine among the spatial sites taken in the stomach. Among these 7 DNA modifications (adducts), there are no adducts preferentially found in the spatial sites of the stomach, where cancer more often occurs. There are some differences of the quantities of these DNA modifications in different locations such as between lesser and greater curvature, proximal and distal, and anterior and posterior walls of the individual stomach, but inter-individual differences were greater than intraindividual (geospatial difference in individual stomach) difference [7].

Life style information and DNA adducts profile: Lifestyle information was taken from the clinical and nursing records of 59 subjects who undertook gastrectomy and 7 autopsy cases who did not have gastric cancer. For smoking, we classified patients into never smokers, current smokers, and former smokers. Estimation of alcohol equivalents was made with reference to the past literature. We collected the frequency and amounts of various alcohol beverages consumed by each individual according to self-reported records, and the total intake was calculated from ethanol equivalents (grams) per drink.

The amount of εdA was greater in smokers than never smokers; in drinkers than never drinkers; and in the subjects who smoke and drink than those who never smoke nor drink. There are few reports on life style information and DNA adducts, then these findings should be corroborated further and mutation signature of tumors in those having relatively large amount of εdA would be intriguing [8].

DNA adductomics in different organs: Multiple DNA adducts profile are reported in several organs using autopsy cases, but the information is still sparse. We investigated DNA adductomics in urothelial mucosa and kidney. The profile of urothelial mucosa was quite different from that of gastric mucosa. For example, lipid peroxidation derived DNA adducts were barely detectable in urothelial mucosa resected for urothelial carcinoma or renal cell carcinoma [9].

Hydroxymethyl cytosine as an epigenetic clock: In both gastrointestinal and urothelial mucosa, 5-hydroxymethyl cytosine in non-tumor mucosa of the stomach or upper urinary tract without cancer are greater than that in non-tumor mucosa of the stomach or upper urinary tract with cancer. Decrease of 5-hydroxymethl cytosine may indicate increased cancer predisposition in these organs [8, 9].

Conclusion: The data on adductomics in human tissue is still scarce especially from the standpoint of prevailing theories such as alkylation mediated carcinogenesis. Refining the methodology is still necessary to provide us definitive evidences toward our long-held understanding of human carcinogenesis. It takes a combination of pathology, epidemiology, and molecular biology in addition to population studies [10, 11].

References

1. Yamagiwa K, Ichikawa K. Exprimentele Studie über die Pathogenese der Epithelialgeschwülste. Mitteilungen Med Fakultät Kaiserl Univ Tokyo 1915; 15:295-344.
2. Lawry PD. Historical Origins of Current Concepts of Carcinogenesis. Adv Cancer Res. 1994; 65:17-111.
3. Hecht SS. Biochemistry, Biology, and Carcinogenicity of Tobacco-Specific N-Nitrosamines. Chem res Toxicol. 1998; 11(6):559-603.
4. Kanaly RA, Hanaoka T, Sugimura H, Toda H, Matsui S, Matsuda T. Development of the adductome approach to detect DNA damage in humans. Antioxid Redox Signal 2006; 8(5-6): 993-1001.
5. Chou PH, Kageyama S, Matsuda S, Kanemoto K, Sasada Y, Oka M, Shinmura K, Mori H, Kawai K, Kasai H, Sugimura H, Matsuda T. Detection of lipid peroxidation-induced DNA adducts caused by 4-oxo-2(E)-nonenal and 4-oxo-2(E)-hexenal in human autopsy tissues. Chem Res Toxicol. 2010; 23(9):1442-8.
6. Matsuda T, Tao H, Goto M, Yamada H, Suzuki M, Wu Y, Xiao N, He Q, Guo W, Cai Z, Kurabe N, Ishino K, Matsushima Y, Shinmura K, Konno H, Maekawa M, Wang Y, Sugimura H. Lipid peroxidation-induced DNA adducts in human gastric mucosa. Carcinogenesis. 2013;34(1):121-7.
7. Iwashita Y, Ohnishi I, Matsushita Y, Ohtsuka S, Yamashita T, Inaba K, Fukazawa A, Ochiai H, Matsumoto K, Kurono N, Matsushima Y, Mori H, Suzuki S, Suzuki S, Tanioka F, Sugimura H. Geospatial Assessments of DNA Adducts in the Human Stomach: A Model of Field Cancerization. Cancers (Basel). 2021;13(15):3728.
8. Ohnishi I, Iwashita Y, Matsushita Y, Ohtsuka S, Yamashita T, Inaba K, Fukazawa A, Ochiai H, Matsumoto K, Kurono N, Matsushima Y, Mori H, Suzuki S, Suzuki S, Tanioka F, Sugimura H. Mass spectrometric profiling of DNA adducts in the human stomach

associated with damage from environmental factors. Genes Environ. 2021;43(1):12.
9. Matsushita Y, Iwashita Y, Ohtsuka S, Ohnishi I, Yamashita T, Miyake H, Sugimura H. A DNA adductome analysis revealed a reduction in the global level of C5-hydroxymethyl-2'-deoxycytidine in the non-tumoral upper urinary tract mucosa of urothelial carcinoma patients. Genes Environ. 2021;43(1):52.
10. Sugimura H. Susceptibility to human cancer: From the perspective of a pathologist. Pathol Int. 2016;66(7):359-68.
11. Natsume H, Szczepaniak K, Yamada H, Iwashita Y, Gędek M, Šuto J, Ishino K, Kasajima R, Matsuda T, Manirakiza F, Nzitakera A, Wu Y, Xiao N, He Q, Guo W, Cai Z, Ohta T, Szekely T, Kadar Z, Sekiyama A, Oshima T, Yoshikawa T, Tsuburaya A, Kurono N, Wang Y, Miyagi Y, Gurzu S, Sugimura H. Non-CpG sites preference in G:C > A:T transition of TP53 in gastric cancer of Eastern Europe (Poland, Romania and Hungary) compared to East Asian countries (China and Japan). Genes Environ. 2023;45(1):1.

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ELUCIDATION OF DRIVER ADDUCTS OF CANCER DEVELOPMENT USING COMPREHENSIVE ANALYSIS OF DNA ADDUCTS

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Humans are exposed to many chemicals in daily life, and it can be assumed that many DNA adducts of exogenous/endogenous origin are generated in our bodies. These adducts are triggers that introduce mutations in the genome, and are therefore considered to be involved in cancer development[1, 2]. However, not all adducts contribute to human carcinogenesis to the same extent. Therefore, it is important to find adducts that contribute to carcinogenesis (driver adducts) to identify carcinogenic factors and risk assessment[3]. With rapid advances in mass spectrometry, several research groups have developed DNA adductome analysis - an emerging method that simultaneously screens for multiple DNA adducts and provides relevant structural information[4-6]. Using this method, we have reported that the carcinogenic mechanism of 1,4-dioxane, a non-genotoxic liver carcinogen, involves DNA adducts derived from inflammatory reactions, and that adducts derived from piperidine, which is contained in black pepper, are a factor in the development of esophageal cancer in China[7, 8].

On the other hand, whole genome/exome sequencing analyses of human cancers have revealed the presence of characteristic mutational profiles, called 'mutational signatures' (https://cancer.sanger.ac.uk/signatures/)[9]. Mutational signatures have been clearly linked with relevant environmental exposure. Furthermore, a strong strand bias observed in a mutational signature suggests that DNA adducts may induce contextual mutations at the relevant regions. These DNA adducts are considered as 'driver adducts' that are significant for human cancer development.

We have analyzed driver adducts of chemicals that have been suggested to be responsible for human cancer development. Occupational exposure to aromatic amines (AAs), such as

o-toluidine (OTD) is an important risk factor for urinary bladder cancer[10]. Recently, ten workers at a Japanese chemical plant handling AAs were diagnosed with bladder cancer[11]. All patients were exposed primarily to OTD and co-exposed to *p*-toluidine, *o*-anisidine, aniline, 2,4-xylidine, acetoacet-*o*-toluidine (AAOT), and *o*-chloroaniline. It has been reported that AAOT can promote urinary bladder carcinogenesis in rats[12]. Thus, OTD and AAOT were considered as the causative agents of occupational bladder cancer in this plant. In the present study, we evaluated the toxicity of AAs and analyzed the carcinogenic mechanisms in rat bladders by comprehensive analysis of DNA adducts (DNA adductome)[13]. DNA was extracted from the bladder epithelia of rats treated with AAs, including AAOT and OTD, and adductome analysis was performed. Principal component analysis–discriminant analysis (PCA-DA) revealed that OTD and AAOT could be clearly separated from the controls and others, including aniline and *p*-toluidine (Figure 1). This finding corresponded with the morphological changes in the rat urinary epithelium, suggesting that DNA adduct formation might contribute to the development of pre-cancerous lesions in the rat urinary epithelium. After confirming the intensity of each adduct, four adducts named adduct32, adduct242, adduct489 and adduct557, were screened as having characteristics of the OTD/AAOT treatment (Figure 2). Comparing with the in-house DNA adduct database, three of four candidates (adduct32, 242 and 489) were identified as oxidative DNA adducts, such as 8-OH-dG, 8-OH-dA and 5-OH-dC based on mass fragmentation together with high-resolution accurate mass (HRAM) spectrometry data (Figure 3)[13]. Therefore, findings suggested that oxidative stress may be involved in the toxicity of rat bladder epithelium exposed to OTD/AAOT. Consequently, another animal experiment determining the effect of

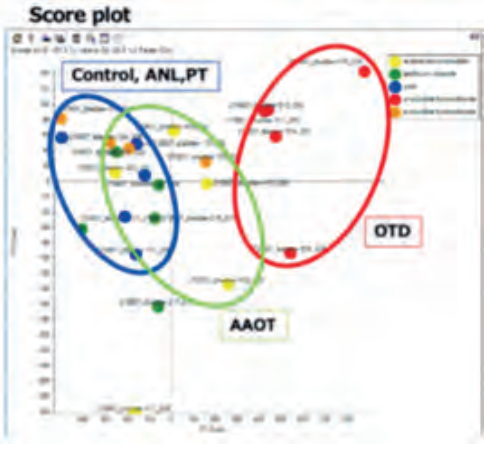

Figure 1 DNA adductome analysis of urinary bladder of rats exposed to AAs.
2D PCA–DA scores of DNA adducts obtained from the adductome analysis. Blue, control; red, OTD; yellow, AAOT; orange, PT; and green, ANL. The clusters are indicated by ellipses: red for OTD, yellow-green for AAOT, and blue for others.

the antioxidant apocynin, an inhibitor of nicotinamide adenine dinucleotide phosphate oxidase, on urinary epithelial hyperplasia induced by OTD was conducted to confirm this hypothesis. Apocynin treatment reduced moderate simple hyperplasia. In addition, labeling indices of Ki67, γ-H2AX, and 8-OHdG in the urinary bladders of rats treated with OTD/apocynin were decreased in a dose-dependent manner compared to that of OTD-treated rats. These findings indicate that oxidative stress may have contributed to the development of urinary cancer induced by OTD.

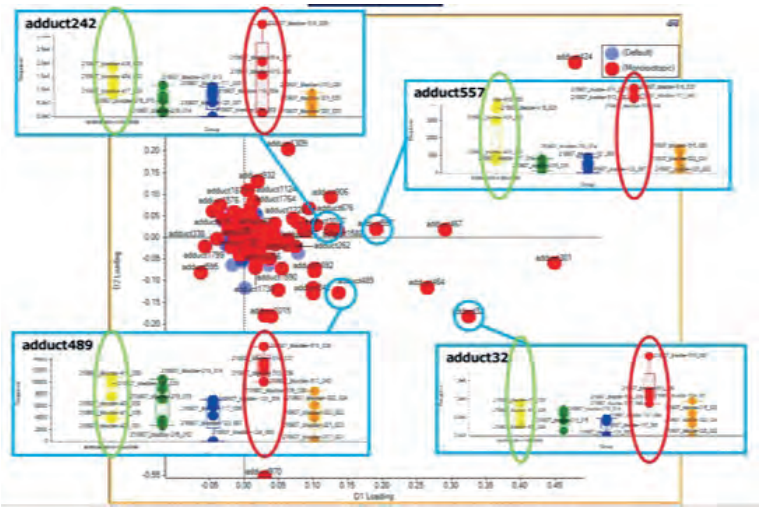

Figure 2 The intensity of four adducts screened as characteristics of the OTD/AAOT treatment. Legends are the same as Figure 1

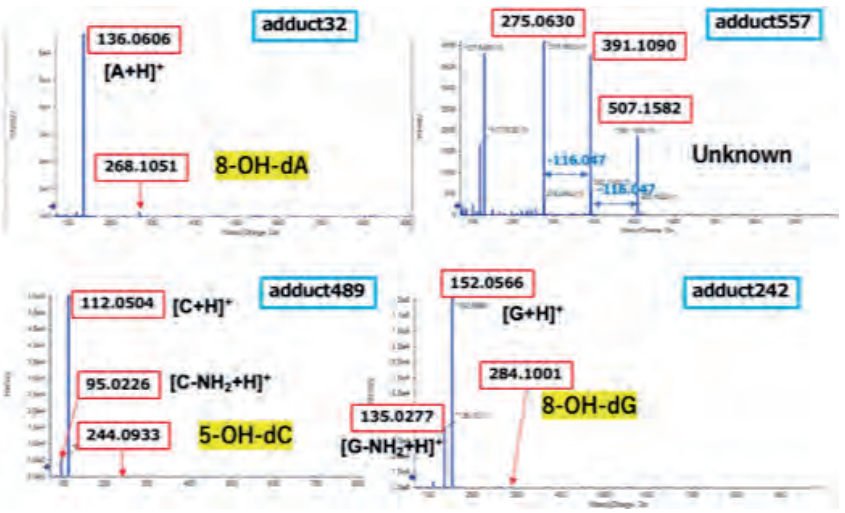

Figure 3 Product ion scan and MS/MS fragmentation data of characteristic DNA adducts for OTD/AAOT.

References

1. Poirier, M.C. (2004) Chemical-induced DNA damage and human cancer risk. *Nature Reviews Cancer*, 4, 630-637.
2. Poirier, M.C. (1997) DNA adducts as exposure biomarkers and indicators of cancer risk. *Environmental Health Perspectives*, 105 (suppl 4), 907–912.
3. Totsuka, Y., Watanabe, M., Lin, Y. New horizons of DNA adductome for exploring environmental causes of cancer. *Cancer Sci*. **2021**, *112*, 7–15.
4. Balbo, S., Turesky, R.J. and Villalta, P.W. (2014) DNA adductomics. *Chemical Research in Toxicology*, 27, 356–366.
5. Kanaly, R.A., Hanaoka, T., Sugimura, H., Toda, H., Matsui, S. and Matsuda, T. (2006) Development of the adductome approach to detect DNA damage in humans. *Antioxidants & Redox Signaling*, 8, 993–1001.
6. Ishino, K., Kato, T., Kato, M., Shibata, T., Watanabe, M., Wakabayashi, K., Nakagama, H. and Totsuka, Y. (2015) Comprehensive DNA adduct analysis reveals pulmonary inflammatory response contributes to genotoxic action of magnetite nanoparticles. *International Journal of Molecular Sciences*, 16, 3474–3492.
7. Totsuka, Y., Maesako, Y., Ono, H., Nagai, M., Kato, M., Gi, M., Wanibuchi, H., Fukushima, S., Shiizaki, K., Nakagama, H. Comprehensive analysis of DNA adducts (DNA adductome analysis) in the liver of rats treated with 1,4-dioxane. *Proc. Jpn. Acad. Ser. B Phys. Biol. Sci*. **2020**, *96*, 180–187.
8. Totsuka Y, Lin Y, He Y, Ishino K, Sato H, Kato M, Nagai M, Elzawahry A, Totoki Y, Nakamura H, Hosoda F, Shibata T, Matsuda T, Matsushima Y, Song G, Meng F, Li D, Liu J, Qiao Y, Wei W, Inoue M, Kikuchi S, Nakagama H, Shan B. DNA adductome analysis identifies *N*-nitrosopiperidine involved in the etiology of esophageal cancer in Cixian, China. 2019, Chem Res Toxicol. 32, 1515-1527.
9. Alexandrov LB, Kim J, Haradhvala NJ, et al. The repertoire of mutational signatures in human cancer. *Nature*. 2020;578:94-101.
10. Cumberbatch, M.G.K.; Noon, A.P. Epidemiology, aetiology and screening of bladder cancer. *Transl. Androl. Urol*. **2019**, *8*, 5–11.
11. Nakano, M.; Omae, K.; Takebayashi, T.; Tanaka, S.; Koda, S. An epidemic of bladder cancer: Ten cases of bladder cancer in male Japanese workers exposed to ortho-toluidine. *J. Occup. Health* **2018**, *60*, 307–311.
12. Yukimatsu, N.; Gi, M.; Okuno, T.; Fujioka, M.; Suzuki, S.; Kakehashi, A.; Yanagiba, Y.; Suda, M.; Koda, S.; Nakatani, T.; et al. Promotion effects of acetoaceto-o-toluidide on N-butyl-N-(4-hydroxybutyl)nitrosamine-induced bladder carcinogenesis in rats. *Arch. Toxicol*. **2019**, *93*, 3617–3631.
13. Suzuki S., Gi M., Komiya M., Obikane A., Vachiraarunwong A., Fujioka M., Kakehashi A.,

Totsuka Y., Wanibuchi H. Evaluation of the Mechanisms Involved in the Development of Bladder Toxicity following Exposure to Occupational Bladder Cancer Causative Chemicals Using DNA Adductome Analysis, Biomolecules, 2024, 14, 36.

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AN ANTHOLOGY OF UNUSUAL PATTERNS OF SOMATIC MUTATIONS IN CANCER GENOMES

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Cancer is the most common human genetic disease with all cancers harboring larger numbers of somatic mutations within their genomes [1]. These mutations are the consequence of the activities of a multitude of mutational processes, including, the intrinsic slight infidelity of the DNA replication machinery, exogenous or endogenous mutagen exposures, enzymatic modifications of DNA, defective DNA repair machinery, and many others [1-3]. In some cancer types, a substantial proportion of somatic mutations are known to be generated by exogenous mutagenic carcinogens, for example, tobacco smoking in lung cancers and ultraviolet light in skin cancers, or by abnormalities of DNA maintenance, for example, defective DNA mismatch repair in certain colorectal and uterine cancers. In other cancer types, such as cancers of the central nervous system, most somatic mutations are due to endogenous mutagenic processes with a significant contribution of the clock-like mutagenic processes that are attributed to normal ageing.

Each mutational process leaves an unusual mutagenic pattern on the genomes of cancer cells, termed, *mutational signature* [1]. Prior computational developments have demonstrated that bioinformatics tools can separate distinct mutational signatures when applied to next-generation sequencing data from cancer samples. We previously developed SigProfilerExtractor, an automated tool for accurate *de novo* extraction of mutational signatures for all types of somatic mutations, that allows a highly sensitive and specific detection of mutational signatures in whole-genome and whole-exome sequenced cancers [4]. Applying SigProfilerExtractor to almost five thousand whole-genome and twenty thousand whole-exome sequenced cancers, encompassing most types of human cancers, has revealed more than 100 distinct mutational signatures with each signature providing significant novel

capabilities that can be leveraged to both better prevent and better treat human cancer [1, 4]. In this extended abstract, by using exemplars, I will briefly present the utility of mutational signatures related to detecting environmental mutagens and to better selecting cancer treatment approaches.

Analysis of cancer genomes has allowed identifying known and unknown mutagens that are linked to environmental exposures and lifestyle choice [1-4]. Notable examples include single base substitution (SBS) signatures SBS4, SBS7, SBS22, and SBS24. Specifically, signature SBS4 is distinguished by C:G>A:T and T:A>A:T mutations, displaying a strong transcriptional strand bias indicative of damage to purine and suggesting a potential interplay with transcription-coupled nucleotide excision repair (TC-NER). This signature is exclusive to cancer types epidemiologically linked to tobacco smoking. Moreover, SBS4 is highly enriched in the genomes of lung cancers from tobacco smokers compared to lifelong non-smokers. SBS7 is identified in cancer types associated with ultraviolet light exposure, such as melanomas and skin squamous cell carcinomas, and it is characterized by C:G>T:A and CC:GG>TT:AA mutations at dipyrimidines. The signature exhibits a pronounced transcriptional strand bias, suggesting the repair of pyrimidine photodimers by TC-NER. SBS22 is primarily marked by T:A>A:T substitutions occurring at Np<u>T</u>pG and Np<u>T</u>pA trinucleotides (mutated base is underlined), displaying a transcriptional strand bias indicative of adenine damage. This mutational signature is exclusively found in cancer samples exposed to aristolochic acid. Signature SBS24 also exhibits a C:G>A:T substitution pattern with a transcriptional strand bias indicative of TC-NER. The mutation patterns of SBS4 and SBS24 differ from each other with signature SBS24 exclusively found in liver cancers from individuals exposed to aflatoxin. Importantly, all four hitherto described mutational signatures have been experimentally validated by exposing cell lines to the respective mutagens. Thus, the characteristic signatures exhibited by different environmental exposures and lifestyle choice allows unambiguously detecting mutagenic exposures. For example, when sequencing liver cancers, one can easily determine whether a specific individual has been consuming products with aflatoxin by the presence or absence of signature SBS24. This type of analysis provides an unprecedented opportunity for detecting previously known and unknown environmental mutagens and for providing mechanistic understanding of epidemiological results related to change in cancer incidence rates across the world.

In addition to detecting environmental mutagens, analysis of unusual patterns of somatic mutations allows a better selection of cancer treatment approaches [3-6]. Failure of DNA repair mechanisms results in the accumulation of specific mutational signatures. Importantly, phenotypically leveraging these mutational footprints of deficiency allows identifying them independent of the mechanism causing the actual DNA repair deficiency. A notable example

for this is signature SBS3. Specifically, SBS3 displays a relatively uniform mutational pattern and has been commonly detected in breast, ovary, pancreas, stomach, and esophageal cancers [1-3]. This signature is linked to inactivating mutations in *BRCA1* and *BRCA2*, crucial components of error-free double-strand break (DSB) repair through homologous recombination. Interestingly, signature SBS3 is also present in numerous samples without mutations in *BRCA1* and *BRCA2*, suggesting that the impairment of DSB repair via homologous recombination might result from BRCA promoter methylation or the inactivation of another gene(s) within the homologous recombination pathway. In line with this observation, SBS3 has been used as a more accurate predictor of the inability to repair DSBs through homologous recombination [5-6], making it a valuable biomarker for predicting response to platinum therapy and PARP inhibitor in breast and ovarian cancer. This example illustrates the utility of mutational signatures as biomarkers for optimally targeting cancer treatment. There are currently at least 11 mutational signatures that reflect failure of DNA repair mechanisms, each of which may provide a potential opportunity for better management of cancer patients in the future.

Overall, the presented anthology of unusual patterns of somatic mutations uncovers the variety of mutational processes that contribute to cancer development, offering insights into the utility of mutational signatures for understanding cancer causes and informing on cancer prevention and better cancer treatment.

References

1. Alexandrov LB, Kim J, Haradhvala NJ, Huang MN, Ng AWT, Wu Y, Boot A, Covington KR, Gordenin DA, Bergstrom EN, Islam SMA, Lopez-Bigas N, Klimczak LJ, McPherson JR, Morganella S, Sabarinathan R, Wheeler DA, Mustonen V, PCAWG Mutational Signatures Working Group, Getz G, Rozen SG, Stratton MR, and PCAWG Consortium (2020) The repertoire of mutational signatures in human cancer. *Nature* 578:94–101.
2. Bergstrom EN, Luebeck J, Petljak M, Khandekar A, Barnes M, Zhang T, Steele CD, Pillay N, Landi MT, Bafna V, Mischel PS, Harris RS, and Alexandrov LB (2022) Mapping clustered mutations in cancer reveals APOBEC3 mutagenesis of ecDNA. *Nature* 602:510–517.
3. Steele CD, Abbasi A, Islam SMA, Bowes A, Khandekar A, Haase K, Hames-Fathi S, Ajayi D, Verfaillie A, Dhami P, McLatchie A, Lechner M, Light N, Shlien A, Malkin D, Feber A, Proszek P, Lesluyes T, Mertens F, Flanagan AM, Tarabichi M, Van Loo P, Alexandrov LB, and Pillay N (2022) Signatures of copy number alterations in human cancer. *Nature*, 606:984–991.
4. Islam SMA, Díaz-Gay M, Wu Y, Barnes M, Vangara R, Bergstrom EN, He Y, Vella M,

Wang J, Teague JW, Clapham P, Moody S, Senkin S, Li YR, Riva L, Zhang T, Gruber AJ, Steele CD, Otlu B, Khandekar A, Abbasi A, Humphreys L, Syulyukina N, Brady SW, Alexandrov BS, Pillay N, Zhang J, Adams DJ, Martincorena I, Wedge DC, Landi MT, Brennan P, Stratton MR, Rozen SG, and Alexandrov LB (2022) Uncovering novel mutational signatures by de novo extraction with SigProfilerExtractor. *Cell Genomics* 2 (11), 100179.

5. Alexandrov LB, Nik-Zainal S, Siu JC, Leung SY, and Stratton MR (2015) A mutational signature in gastric cancer suggests therapeutic strategies. *Nature Communications*, 6 (article# 8683).
6. Tymon-Rosario JR, Manara P, Manavella DD, Bellone S, Hartwich TMP, Harold J, Yang-Hartwich Y, Zipponi M, Choi J, Jeong K, Mutlu L, Yang K, Altwerger G, Menderes G, Ratner E, Huang GS, Clark M, Andikyan V, Azodi M, Schwartz PE, Alexandrov LB, and Santin AD (2022) Homologous recombination deficiency (HRD) signature-3 in ovarian and uterine carcinosarcomas correlates with preclinical sensitivity to Olaparib, a poly (adenosine diphosphate [ADP]- ribose) polymerase (PARP) inhibitor. *Gynecologic Oncology*, 166 (1), 117-125.

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TRANS-ANCESTRY MUTATIONAL AND STRUCTURAL ALTERATION SPECTRUM LANDSCAPE OF GASTRIC CANCER

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Gastric cancer (GC) is the third most common cancer killer worldwide. Its incidence varies from region to region. The highest rates are found in East Asia, Eastern Europe and South America, and the lowest in North America. In addition to germline variants, a number of environmental risk factors have been reported, including chronic infection with Helicobacter pylori (HP) or Epstein-Barr virus (EBV), and lifestyle factors such as salt intake, alcohol consumption and smoking. However, no comprehensive molecular study has been conducted to uncover the somatic drivers associated with these geographic and epidemiological differences. Diffuse gastric cancer, a subtype of GC with a poor prognosis, still has low five-year survival rates and the development of more effective treatment and prevention methods is expected. The diffuse type accounts for about 30% of cases, in which eradication of H. pylori has little preventive effect. Carcinogenic exposures may differ between races, and international collaborations can facilitate the discovery and study of ethnic-specific carcinogens by comparing somatic signature profiles.

1. Trans-ethnic mutational landscape of gastric cancer

We have reported a comprehensive, multi-ancestral landscape of driver events in the largest (more than 1400 cases) gastric cancer (GC) cohort, including 697 Japanese cases, 328 Caucasian cases, 391 cases from East Asian regions (China, Korea, Singapore and Vietnam) and 41 cases from other areas[1]. Our mutational signature analysis revealed that different mutational processes were represented by mutational signatures between subtypes, EBV infection and ancestry groups. In particular, COSMIC-Sig16 contributed more frequently to diffuse type cases (P=0.0010), East Asian ancestry (P=2.9e-5) and alcohol consumption

(P=4.8e-6). Germline variations in alcohol dehydrogenase 1 B (*ADH1B*, rs1229984 AA/AG) and aldehyde dehydrogenase 2 (*ALDH2*, rs671 AA/AG) were also significantly associated with higher COSMIC-Sig16 contribution (P=8.8e-5 and 2.2e-16, respectively).

We compared the sequence contexts of recurrent identical mutations with those of COSMIC signatures. Characteristically, the hot-spot mutation context of *RHOA*, a hallmark diffuse-type driver gene, was similar to that of COSMIC-Sig16. *RHOA* Y42C hot-spot mutations were significantly enriched in the COSMIC-Sig16 high cases (P=3.0e-5) and alcohol drinking with *ADH1B*/*ALDH2* germline variants (P=0.011, OR=5.4, P=0.0021, OR=6.9, respectively) (Figure 1). These results demonstrate that germline variants (*ADH1B*/*ALDH2*) and lifestyle (alcohol consumption) synergistically contribute to the acquisition of specific driver mutations (*RHOA* Y42C) and may partly explain the higher incidence of diffuse-type GCs in the East Asian population.

2. Structural alteration spectrum of gastric cancer genomes

The assessment of non-coding region driver aberrations, such as extrachromosomal DNA (ecDNA) and complex chromosomal structural aberrations, is still inadequate and their patterns and processes remain poorly understood. To explore the molecular and epidemiological background of structural variation (SV) accumulation processes, we examined 170 GC whole genomes and extracted SV signatures representing SV propensity[2]. We identified six rearrangement signatures (RS). Unsupervised hierarchical clustering

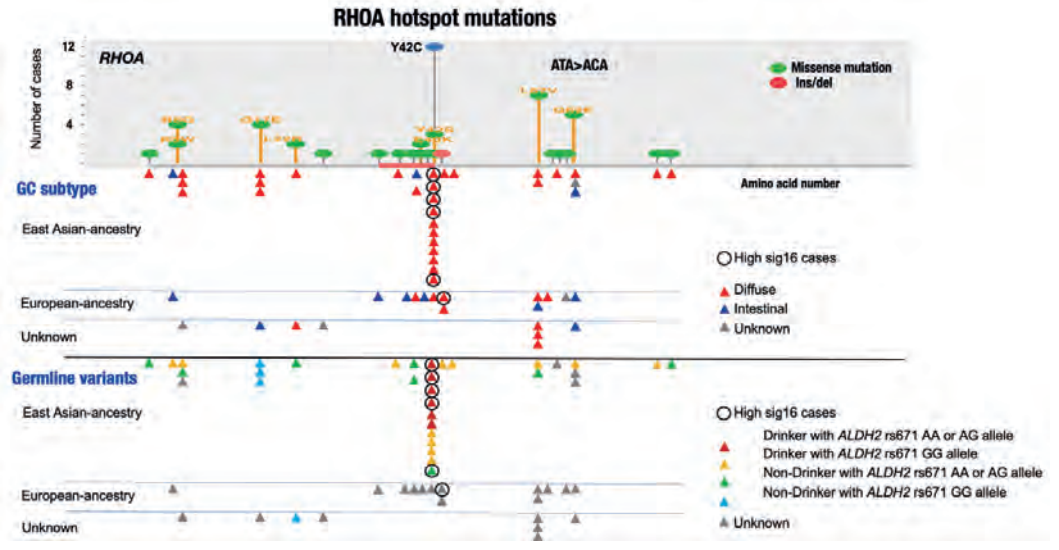

Figure 1 RHOA Y32C mutation was enriched with East-Asian ancestry, diffuse-type, drinkers with ALDH2 rs671 AA/GG allele cases with high COSMIC Sig16.

classified 170 cases into seven RS subtypes (subtype RS1-6 and subtype RS2/6); non-random combinations of RSs revealed unique GC subtypes with one or a few dominant RSs associated with unique driver events (*BRCA1/2* defects, mismatch repair deficiency and *TP53* mutation) and epidemiological backgrounds.

SV clusters (SVCs) were frequently detected in cancer genomes and are associated with oncogene amplification. 36.6% of the total SVs were clustered, forming 3,457 SVCs in GC genomes. We then annotated the proposed molecular mechanisms: CFS, L1 retrotransposition, non-allelic homologous recombination-mediated duplication, and breakage-fusion-bridge cycles. Our analysis also identified other complex types of SVCs that were not classified as described above. They exhibited high SVAF breakpoints at the amplicon edges and contained a group of lower SVAFs densely located within the amplicon. In some cases, these amplified segments were joined over distances > 2 Mb. We termed the former a self-joining amplicon and the latter an assembled amplicon, both of which shared with similarities to ecDNA (Figure 2).

The chromosomal distribution of these SVCs with amplicon types revealed that they were enriched in specific genomic regions containing GC oncogenes. In addition, previously reported ecDNA-associated amplicon loci overlapped with self-joining/assembled amplicon SVCs. In addition to GC oncogene loci, we discovered SVC-enriched amplified loci, such as 1q42.3, 13q34 and 18q12-13, where the target genes remain to be determined. Among these, the 1q42.3 genomic fragment has been reported to induce cellular senescence, suggesting the

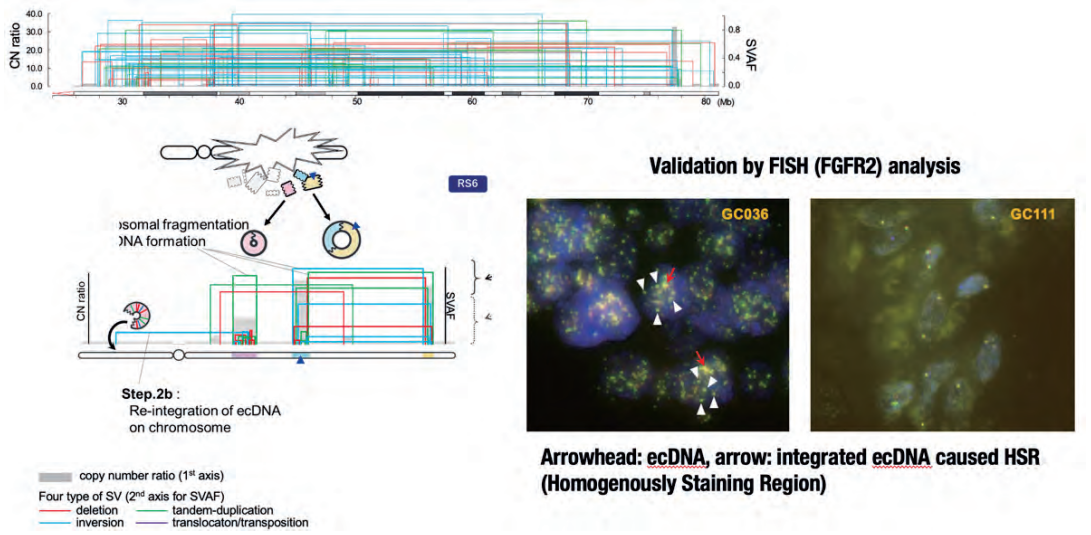

Figure 2 Unique SV patterns are associated with extra-chromosomal DNA (ecDNA)

existence of an unexplored oncogene.

References

1. Totoki Y, Saito-Adachi M, Shiraishi Y, Komura D, Nakamura H, Suzuki A, Tatsuno K, Rokutan H, Hama N, Yamamoto S, Ono H, Arai Y, Hosoda F, Katoh H, Chiba K, Iida N, Nagae G, Ueda H, Shihang C, Sekine S, Abe H, Nomura S, Matsuura T, Sakai E, Ohshima T, Rino Y, Yeoh KG, So J, Sanghvi K, Soong R, Fukagawa A, Yachida S, Kato M, Seto Y, Ushiku T, Nakajima A, Katai H, Tan P, Ishikawa S, Aburatani H, Shibata T. Multiancestry genomic and transcriptomic analysis of gastric cancer. *Nat Genet*. 2023, 55, 581-594.
2. Saito-Adachi M, Hama N, Totoki Y, Nakamura H, Arai Y, Hosoda F, Rokutan H, Yachida S, Kato M, Fukagawa A, Shibata T. Oncogenic structural aberration landscape in gastric cancer genomes. *Nat Commun*. 2023, 14, 3688.

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CAN WE IDENTIFY NEW CAUSES OF CANCER THROUGH LARGE SCALE GENOMIC STUDIES ?

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International differences in the incidence of many cancer types indicate the existence of carcinogen exposures that have not been identified by conventional epidemiology yet potentially make a substantial contribution to cancer burden[1]. Some carcinogens generate somatic mutations and, therefore, a complementary strategy for detecting past exposures is to sequence the genomes of cancers from populations with different incidence rates inferring underlying causes from differences in patterns of somatic mutations. As an example of this approach, we sequenced 962 renal cancers from 11 countries of varying incidence. Somatic mutation profiles differed between countries. In Romania, Serbia and Thailand, mutational signatures likely caused by extracts of Aristolochia plants were present in most cases and rare elsewhere. In Japan, a mutational signature of unknown cause was found in >70% cases and <2% elsewhere. A further mutational signature of unknown cause was ubiquitous but exhibited higher mutation loads in countries with higher kidney cancer incidence rates (p-value $<6\times10^{-18}$). Known signatures of tobacco smoking correlated with tobacco consumption, but no signature was associated with other known causes of renal cancer including obesity or hypertension, suggesting non-mutagenic mechanisms of action underlying these risk factors. The results indicate the existence of multiple, geographically variable, mutagenic exposures potentially affecting 10s of millions of people and illustrate the opportunities for new insights into cancer causation through large-scale global cancer genomics.

References

1. Brennan, P. & Davey-Smith, G. Identifying Novel Causes of Cancers to Enhance Cancer Prevention: New Strategies Are Needed. *JNCI: Journal of the National Cancer Institute* **114**, 353–360 (2022).

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MUTATIONAL SIGNATURES OF CHEMOTHERAPEUTIC AGENTS AND ENVIRONMENTAL CARCINOGENS

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Reporter gene assays, in which a single mutation from each experiment can contribute to the assembly of a mutation spectrum for an agent, have provided the basis for understanding the mutational processes induced by mutagenic agents and for providing clues to the origins of mutations in human tumours[1]. More recently exome and whole genome sequencing of human tumours has revealed distinct patterns of mutation that provide additional clues for the causative origins of cancer. These can be tested by examining the mutational signatures induced in experimental systems by putative cancer-causing agents. The generation of multiple mutations per experiment represents a distinct advantage over single gene assays, which generally produce one mutation per experiment.

We have investigated mutational signatures in transformed mouse embryo fibroblast cells[2], in human induced pluripotent stem cells[3] (hIPSCs) and in human tissue organoid cultures. Proof of principle has established that mutational signatures generated in mouse cells by simulated sunlight and aristolochic acid matched those signatures found in human melanomas and urothelial cancers, respectively[2]. Benzo[a]pyrene produced a signature that is a close match to the smoking-related signature detected in the respiratory tract tumours of tobacco smokers[2]. Each DNA sample was capable of generating up to tens of thousands of mutations with which to construct the mutational signatures. In hIPSCs, of 79 agents tested with or without metabolic activation at concentrations that produced measurable cytotoxicity, 41 yielded characteristic single-base substitution (SBS) mutational signatures[3]. Several exhibit similarity with signatures found in human tumours, including those associated with simulated sunlight, aristolochic acid and benz0[a]pyrene already observed in mouse cells. Additionally, 6 agents produced double-base substitution (DBS) signatures

and 8 produced indel (ID) signatures. Investigating mutation asymmetries across genome topography revealed fully functional mismatch and transcription-coupled repair pathways in iPSCs, the latter evident as strand bias in mutations.

In human tissue organoids there was a lesser requirement for an exogenous metabolizing system, as the cultures were, in most cases, capable of activating test agents[4, 5]. The application of duplex sequencing has removed the need for cloning of mutated cells, as sequencing both DNA strands enables errors in PCR amplification are eliminated from the signatures. The signatures for benzo[*a*]pyrene (BaP), aflatoxin B1 (AFB1), aristolochic acid (AA1) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) were qualitatively similar in five different human tissue organoids – gastric, colon, liver, pancreas and kidney – but there were quantitative differences. The mutational signatures of a further 16 environmental carcinogens and 30 cancer chemotherapy agents have been investigated in human gastric organoids, providing additional insights into processes underlying the causes of human cancer.

References

1. Phillips, D.H. Mutational spectra and mutational signatures: insights into cancer aetiology and mechanisms of DNA damage and repair. *DNA Repair*, **71**, 6-11 (2018).
2. Nik-Zainal, S., Kucab, J.E., Morganella, S., Glodzik, D., Alexandrov, L.B., Arlt, V.M., Weningen, A., Hollstein, M., Stratton, M.R. and Phillips, D.H. The genome as a record of environmental exposure. *Mutagenesis*, **30**, 763-770 (2015).
3. Kucab, J.E., Zou, X., Morganella, S., Joel, M., Nanda, A.S., Nagy, E., Gomez, C., Degasperi, A., Harris, R., Jackson, S.P., Arlt, V.M., Phillips, D.H. and Nik-Zainal, S. A compendium of mutational signatures of environmental and chemotherapeutic agents. *Cell*, **177**, 821-836 (2019).
4. Caipa Garcia, A.L., Kucab, J.E., Al-Serori, H., Beck, R.S.S., Fischer, F., Hufnagel, M., Hartwig, A., Floeder, A., Balbo, S., Francies, H., Garnett, M., Huch, M., Drost, J., Zilbauer, M., Arlt, V.M. and Phillips, D.H. Metabolic activation of benzo[a]pyrene by human tissue organoid cultures. Int. *J. Mol. Sci*., **24**, e606 (2023).
5. Caipa Garcia, A.L., Kucab, J.E., Al-Serori, H., Beck, R.S.S., Bellamri, M., Turesky, R., Groopman, J., Francies, H., Garnett, M.J., Huch, M., Drost, J., Zilbauer, M., Arlt, V.M. and Phillips, D.H. The use of human tissue organoid cultures to study the metabolic activation of dietary carcinogens. *Chem. Res. Toxicol*., in press (2023).

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ENVIRONMENTAL CARCINOGENESIS, MUTATIONAL SIGNATURES AND RATIONAL DRUG DESIGN

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The *Aristolochiaceae* family of herbaceous plants has been utilized for medicinal purposes for more than 2500 years. Toxicologists have long been aware of its toxic properties. The dramatic revelation that aristolochic acid (AA) is a powerful nephrotoxin and human carcinogen drew attention to the extensive use of *Aristolochia sp*. worldwide. Twenty years or more may elapse before AA-induced renal dysfunction becomes apparent or AA-induced tumors develop. Some AA-DNA adducts are remarkably stable, having been detected decades after exposure and lead to A:T → T:A mutations. Mutational analysis of the p53 tumor suppressor gene in upper urothelial cancers from Balkan Endemic Nephropathy regions and from Taiwan, two area with high exposure to *Aristolochia*, revealed that the frequency of A:T → T:A mutations was 78%. The genome-wide mutational signature for AA has become a powerful tool for dissecting the epidemiology of AA-related pathologies. This signature has been detected in healthy tissues and in tumors from patients with many cancer types including upper tract cancer, bladder cancer, renal cell cancer, liver cancer, and intrahepatic cholangiocarcinoma.

We, in collaboration with Dr. Robert Turesky of University of Minnesota and Dr. Chung-Hsin Chen of National Taiwan University, are developing "adductomic" techniques to both simultaneously identify the totality of long-lived bulky DNA-adducts in a patient DNA sample and to determine the relationship of those adducts to mutational signatures in the DNA sample. Aristolochic acid derived adducts serve as a valuable "internal control" expected to be found in patient samples from Taiwan. To date, only aristolochic acid I derived adducts of dA have been definitively linked to human disease. But several other AA species exist in most *Aristolochia*. Dr. Turesky's adductomic techniques has detected the dG

and dC aristolactam-adducts derived from AAI. Also, for the first time, in rat DNA, he has detected the dA adducts derived from AAIII, a common AA in *Aristolochia*. In the rat kidney, AAIII forms adducts 30-fold more efficiently than AAI.

In the endemic regions of the Balkans contamination of feedstocks by *Aristolochia* is the route of exposure to AA. Modern technology can be used to confirm the elimination of *Aristolochia* from agricultural fields. We are using remote sensing with high-resolution multispectral cameras mounted on robotic drones. The images obtained are utilized for the detection and precise localization of *Aristolochia* plants within wheat fields wherever home-made bread is a principal source of food.

Following bioactivation in the liver, unmodified AAs and their active species are transported via circulation and elicit carcinogenic effects by binding to DNA of various tissues. We apply x-ray crystallography, DNA adduct analysis, fluorescence quenching and isothermal titration calorimetry to elucidate the role of human serum albumin (HSA) in AA's carcinogenicity. These structural and thermodynamic analyses of AA-HSA interactions yield significant insight into the binding, transport, toxicity and potential allostery of AAs. The crystallographic data obtained for binding to HSA at 1.9A resolution is amongst the highest resolution structures available for such ligand protein complexes.

In a separate venture we are repurposing classic medicines as antivirals to face modern challenges such as the recent COVID-19 pandemic. Emetine has been used in the clinic for the past century to treat amoebiasis and as a broad-spectrum antiviral agent. However, at the doses required as an anti-amoebic, emetine has significant side-effects that lead to its replacement over time. But emetine is effective against coronaviruses at 100-fold lower doses than required for treating amoebic dysentery. Hence, we are initiating a clinical trial to test the effectiveness of emetine as an anti-CoV-2 agent.

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THE INTERPLAY BETWEEN ENVIRONMENTAL MUTAGENS AND INFLAMMATORY TUMOUR PROMOTING FACTORS IN DETERMINING CANCER RISK.

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Somatic point mutations in cancer driver genes are essential for the development of most cancers, but deep sequencing studies have demonstrated that similar mutations can accumulate in normal tissues during the ageing process. These observations have raised many questions regarding the mechanisms that allow normal tissues to tolerate the presence of cells carrying potent mutations, and the factors that stimulate the emergence of neoplastic clones. In particular, the relative contributions of mutations and exogenous environmental tumour promoters or endogenous processes related to chronic tissue damage, inflammation, and obesity, to cancer risk are presently unclear and highly controversial. We have shown, using mouse tumour models (1) that many known or suspected human carcinogens do not induce a high mutational burden, or novel mutational signatures, thus raising the possibility that the role of promoting factors in cancer risk has been underestimated. Moreover, recent work from the Swanton laboratory has also demonstrated that environmental pollutants such as air particulates may promote "initiated", dormant pre-cancer cells to develop into lung cancers, for the first time explaining why non-smokers may develop EGFR mutated lung adenocarcinomas (2).

In order to quantify the relative contributions of mutations and promoting factors to cancer risk, we have replicated the seminal studies of mouse multistage skin cancer development carried out in the 1940s (3) suggesting that "initiated cells" are capable of remaining dormant in normal mouse tissue for most of the mouse lifespan, but can be promoted to form visible tumours at any time by repeated exposure to an irritant, tumour promoting plant extract. In spite of the importance of these historic studies in formulating present models of multistage carcinogenesis, and in demonstrating the critical role of the

promotion stage in cancer risk, the primary observations have never been replicated in the modern era and examined using next generation sequencing to quantify the numbers of initiating mutations induced by carcinogens, to demonstrate their persistence in normal tissue for long periods of time, and their continued responsiveness to tumour promoters. Similarly, although classical tumour promoters are widely assumed to be non-mutagenic as they fail to induce point mutations in bacterial assays such as the Ames test, the possibility that irritant and promoting factors may contribute to increased mutation burden indirectly, through activation of oxidative stress, DNA damage, or gross chromosomal alterations has never been adequately investigated.

To address these unanswered questions, we carried out two series of experiments to a) test the hypothesis that chemically-induced tumours arise from long lived stem cells in the mouse skin, and b) determine how many mutations are induced by a single initiating dose of a mutagen (DMBA), and demonstrate that cells carrying these mutations lie dormant in the skin for most of the mouse lifespan without causing any obvious pathology. To address the first question, we carried out studies using reporter mice that enabled us to track the stem cell population(s) that were capable of initiation in vivo using exogenous mutagens and tumour promoters (4). These studies led to the conclusion that chemically initiated tumours arise from long-lived stem cells in the upper hair follicle rather than from the classical bulge stem cell region, and furthermore confirmed the prediction by Berenblum and Shubik (3) that the most important and rate-limiting step in carcinogenesis was exposure to a promoting factor rather than initiation. To address the second question, we carried out whole genome sequencing (WGS) of over 100 mouse skin tumours induced by a range of mouse models involving initiation by exposure to potent mutagens or genetic induction of *Hras* or *Kras* mutations, followed by repeated exposure to strongly inflammatory processes such as tissue damaging agents, wounding, and obesity. We also examined the permanence of mutations induced by carcinogen exposure either during fetal development or in adult mice, followed by a long latency period of 6-12 months before treatment with a tumour promoter (5).

Taken together, our results suggest that a single mutagenic exposure at any point in an individual's lifespan can result in sufficient mutations, in the correct combinations, for initiation of carcinogenesis, and that these cells are not removed by the immune system or competition with normal cells during embryonic development, nor during an extended period of adult life. We also conclude that the rate-limiting determinant of early tumour growth is exposure to a tumour promoter or tissue injury, rather than the number of genomic mutations. Clearly, mutations in cancer driver genes are essential for tumours to develop, but the process of promotion is also essential to stimulate the outgrowth and clonal selection of cells carrying these driver mutations. The constant stimulation of wound healing

responses by tumour promoters leads to chromosome changes that have been noted previously by cytological and genetic analysis of skin tumours (6, 7), and further selection of specific chromosome changes may help to fix certain cell states essential for advancement through the pre-malignant stage and progression to malignancy (8).

These considerations help us to reconcile various models of cancer initiation and progression as being primarily due to (A) sequential accumulation of the point mutations necessary to activate the various cancer "hallmark" properties, or (B) induction or spontaneous occurrence of one or very few cancer driver mutations that are adequate for genetic initiation, but require repeated exposure to agents that cause tissue damage in order to undergo clonal expansion and ultimately progression to malignancy (9). It is likely that large scale chromosomal events (aneuploidy, loss of heterozygosity or deletions) can help to enable progression through the biological stages of pre-malignancy until cells arise that have become independent from continued promoter treatment, and emerge as fully malignant clones (Fig.1)

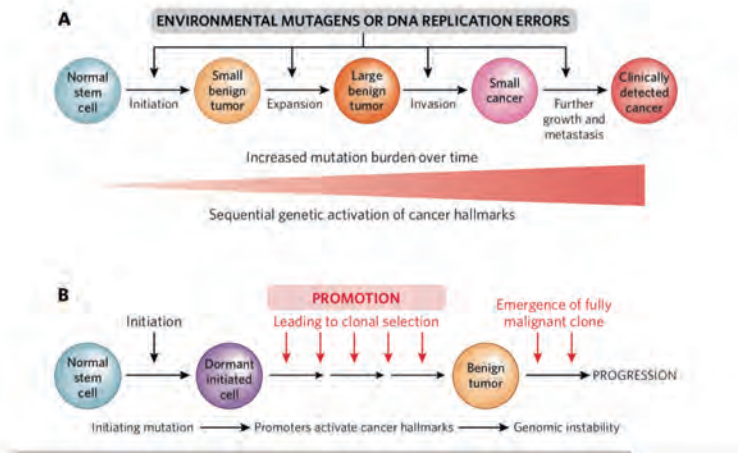

Figure 1 A (9): The sequential mutation model for cancer development compatible with the original age-incidence data from Armitage and Doll suggesting around 6 "stages", each associated with a mutational event, and acquisition of cancer hallmarks such as self sufficiency in growth factors, resistance to cell death, recruitment of new blood vessels (angiogenesis), and ability to invade and metastasize.

B: The two stage clonal selection model, originally developed using mouse models (3), by which initiation is accomplished by a single treatment with an initiator, generating dormant mutated cells that are dependent on exposure to a tumour promoting agent or wounding in order to clonally expand. Tissue regeneration caused by TPA or wounding induces many of the cancer hallmark properties including increased growth factor production, angiogenesis, invasive behaviour, and immuno-evasion. Chronic treatment with a promoter is necessary to ensure clonal expansion, and initiated cell clones lose their competitive advantage if promotion is stopped. Progression through these stages of pre-neoplasia and malignant development involves acquisition of independence from continued promoter treatment, through genomic alterations that lead to genetic instability and loss of tumour suppressor gene activity.

Further studies of the exact mechanisms of tumour promotion can have major implications for development of new strategies for cancer prevention.

References

1. Riva, L. *et al*. The mutational signature profile of known and suspected human carcinogens in mice. *Nat Genet* 52, (11):1189-97 (2020).
2. Lung adenocarcinoma promotion by air pollutants.
   Hill W, et al. Nature. 2023. PMID: 37020004
3. Berenblum I, Shubik P. The persistence of latent tumour cells induced in the mouse's skin by a single application of 9:10-dimethyl-1:2-benzanthracene (1949). Br J Cancer 3(3):384-6.
4. Kandyba, E. *et al*. Chemically-induced skin tumours arise from long-lived stem cells of the upper hair follicle. Submitted.
5. Yun Rose Li et al, The interplay between dormant mutated cells and tumor promotion by chronic tissue damage in determining cancer risk. Submitted
6. Aldaz CM, Trono D, Larcher F, Slaga TJ, Conti CJ. Sequential trisomization of chromosomes 6 and 7 in mouse skin premalignant lesions.
   Mol Carcinog. 1989;2(1):22-6. doi: 10.1002/mc.2940020104.PMID: 2499343
7. Bremner R, Kemp CJ, Balmain A. Induction of different genetic changes by different classes of chemical carcinogens during progression of mouse skin tumors. Mol Carcinog. 1994 Oct;11(2):90-7. PMID: 7916997
8. Taylor, M. et al Gene networks reveal stem-cell state convergence during preneoplasia and progression to malignancy in multistage skin carcinogenesis. Submitted.
9. Balmain A. Peto's paradox revisited: black Box vs mechanistic approaches to understanding the roles of mutations and promoting factors in cancer. Eur J Epidemiol. 2022 Dec 13. doi: 10.1007/s10654-022-00933.PMID: 36512199

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CARBON NANOTUBES, INNOVATIVE MATERIALS OF THE 21st CENTURY: CARCINOGENICITY EVALUATION OF CNTs WITH DIFFERENT WALL STRUCTURES

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Carbon nanotubes (CNT) are composed of concentric one-atom thick graphene cylinders. The carbon-carbon bonds of graphene are exclusively sp2, giving CNTs remarkable chemical and physical properties. Consequently, CNTs have numerous industrial applications and the elimination of currently manufactured CNTs would likely have a significant negative impact. However, the light weight and stability of CNTs also give rise to concern regarding potential human toxicity. Therefore, hazardous CNTs need to be identified so that appropriate safety precautions can be put into place that ensure the safe manufacture and use of these extremely valuable materials.

In spite of the potential hazard to human health presented by respirable CNTs, very few long term studies of CNTs administered into the lung have been carried out. In October, 2014, IARC assessed the carcinogenicity of CNTs. They concluded that there was sufficient evidence in experiment animals to classify multi-walled CNT-7 (MWCNT-7; also known as Mitsui-7, MWNT-7) as possibly carcinogenic to humans (group 2B), but that there was inadequate evidence in experimental animals to classify the carcinogenicity of single-walled CNTs (SWCNTs) and MWCNTs other than MWCNT-7, placing all CNTs except for MWNCT-7 as not classifiable as to their carcinogenicity to humans (Group 3) [1]: the final report was published in 2017 [2]. Notably, at the time of the IARC assessment of CNTs, not a single 2-year carcinogenic study of CNTs administered into the lung had been published.

Whole-body inhalation studies are extremely complex [3], and there are very few facilities equipped to carry out such studies. At the time of this writing, only a single 2-year study of CNTs administered by inhalation has been carried out [4]. Therefore, a reliable method that administers respirable CNTs to the lungs that reflects potential human exposure using

techniques and equipment that are more commonly accessible to research groups needs to be used for the testing of the many different types of CNTs being produced. We have established "intra-tracheal, intra-pulmonary spraying (TIPS)" as a method for the administration of CNTs evenly throughout lung using readily available techniques and equipment [5].

Summary of 2-Year Carcinogenicity Studies of CNTs Administered to the Lung

In our studies MWCNTs were administered to rats eight times (total 0.5~1.5 mg/rat) over a 2-week period. The rats are then observed without any further treatment for up to 2 years. We initially assessed the carcinogenicity of MWCNT-N, a thick straight MWCNT with 40 wall layers [6]. We found that these fibers induced lung tumors and pleural malignant mesotheliomas. This was the first study to show the carcinogenicity of an MWCNT after administration into the lung. Next, a very thick, straight MWCNT, MWCNT-A (150 layers), and a thin tangled MWCNT, MWCNT-B (15 layers), were tested [7]. Contrary to expectation, we found that MWCNT-B was more carcinogenic to the lung than MWCNT-A. In this study, 3 rats administered 1.0 mg MWCNT-A developed lung carcinomas while no rats administered vehicle developed lung carcinomas and no rats administered crocidolite asbestos (a known human lung carcinogen) developed lung carcinomas, suggesting that while the induction of lung carcinomas in the rats administered MWCNT-A was not statistically significant, MWCNT-A likely has carcinogenic potential. We then tested a far thinner CNT, double-walled CNT (DWCNT), and found that DWCNT was also carcinogenic to the lung [8]. These studies indicate that CNT carcinogenicity in the lung is not restricted to long, straight CNTs.

The Japan Bioassay Research Center (JBRC) published the results of its 2-year inhalation exposure to MWCNT-7 in 2016 [4]. This study found that inhalation of MWCNT-7 induced lung tumors, but unlike MWCNT-N, inhalation of MWCNT-7 did not induce mesothelioma development. However, in a study by Numano et al., instillation of MWCNT-7 at 1.5 μg/rat resulted in development of mesothelioma in 18 of 19 rats, the other rat died from a pituitary tumor. The amount of MWCNT-7 fibers in the lungs of the male rats exposed to 2 mg/m^3 MWCNT-7 at the end of 2 years in the JBRC study was 1.8 mg and the instilled dose of MWCNT-7 in the Numano et al. study was 1.5 mg, suggesting that if the rats in the JBRC study had survived for an addition 1 to 2 years, they would have developed mesotheliomas, while in the Numano et al. study, if the rats had not developed malignant mesothelioma, they would have developed lung tumors. A study by Hojo et al, supports these arguments. Hojo et al, administered MWCNT-7 by instillation into the lung once every 4 weeks for 2 years [9]. They found that MWCNT-7 induced both lung tumors and mesothelioma.

Overall, administration of thick straight MWCNT-N and MWCNT-7 resulted in lung

tumor and mesothelioma development, while administration of thin tangled MWCNT-B and DWCNT resulted in lung tumor development but not mesothelioma development.

A Brief Discussion of Mechanism

Inflammation was assessed in four of the two year studies cited above. Exposure to MWCNT-7, MWCNT-B, and DWCNT caused chronic inflammation [4, 7, 8, 9]. In a recent study, rats were administered MWCNT-7, MWCNT-N, MWCNT-B, and DWCNT, and lung tissue was collected 6 weeks after administration and RNA sequences were analyzed. Analysis of the up and down-regulated RNAs was consistent with inflammatory signaling in the lung tissues of the CNT treated rats [Data presented at the PTCRF International Symposium, manuscript in preparation]. These findings are in agreement with those of a study by Hojo et al. that found that MWCNT-7 induced tumorigenesis in the lung was consistent with inflammation-induced carcinogenesis [10].

Importantly, macrophages were able to completely engulf the MWCNT-N, MWCNT-7, MWCNT-A, MWNCT-B, and DWCNT fibers that we tested (Fig. 1). However, all these fiber types persisted in the lung. In agreement with these findings, the inhalation studies conducted by JBRC using MWCNT-7 also demonstrated that fibers that were completely engulfed by macrophages persisted in the lung, resulting in chronic inflammation and lung tumor development. Harmful fibrous particles like asbestos are known to induce inflammation via their interaction with macrophages. Fibers that cannot be removed from the lung cause repeated cycles of inflammation-induced tissue damage which is followed by tissue repair. These cycles of tissue damage and repair can cause DNA mutations that can be fixed in daughter cells. One of the primary mechanisms by which non-degradable fibers are retained in the lung is thought to be the inability of macrophages to completely engulf long fibers resulting in frustrated phagocytosis. This led to the assumption that short fibers may

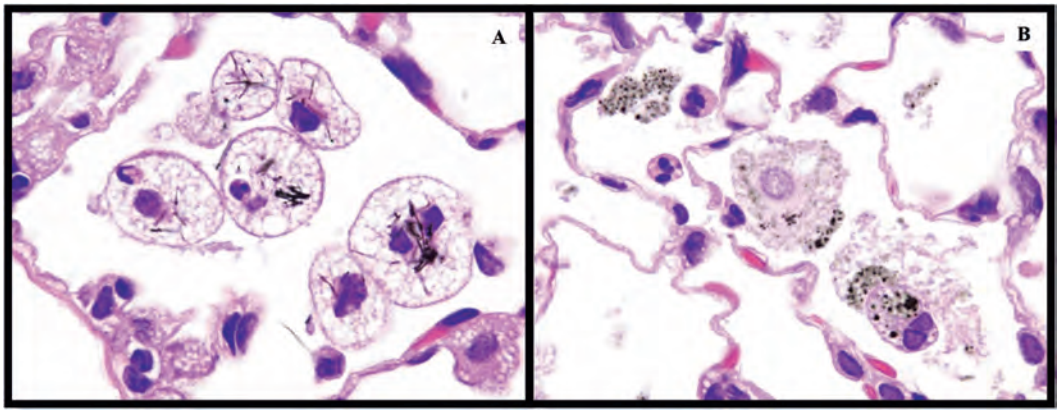

Figure 1 (A) MWCNT-A > 100 layers (Ø150 nm). (B) MWCNT-B 15 layers (Ø7.4 nm).

not be toxic. However, it is now clear that removal from the lung of MWCNTs that are short and flexible enough to be completely engulfed by macrophages can also impeded, possibly by generation of a "STOP" signal [3]. Consequently, short CNTs can also be carcinogenic.

Overall, carcinogenic CNTs have two effects on alveolar macrophages. First, phagocytosis of the CNTs by macrophages results in inflammatory signaling. Second, the phagocytosed CNTs impede the ability of the macrophages to remove the CNT from the alveoli: CNTs impede the ability of alveolar macrophages to sequester the CNTs in granulation tissue and they also impede the macrophage from physically removing the CNTs out of the alveoli into the ciliated airways. These two effects result in continuous inflammatory signaling and production of cytotoxic molecules that will result in the death of the macrophage and release of the CNTs, which are then phagocytosed by other macrophages attracted to the site of inflammation. This results in the chronic inflammation, tissue damage and tissue repair, DNA mutations, and the eventual development of tumors reported by the studies cited above.

Importantly, the induction of tissue damage and DNA mutations in the lung differs from that in the pleural cavity. In the lung, interaction of biopersistent CNTs with macrophages leads to chronic inflammation and tissue damage and DNA mutations. In contrast, intraperitoneal injection studies identified CNTs that induced the development of mesotheliomas as being straight (acicular) with low levels of agglomerate formation [11, 12, 13]. These CNTs were cytotoxic to mesothelial cells and it was concluded that direct injury to mesothelial cells was responsible for the induction of inflammation and the subsequent development of mesotheliomas. In contrast, thin tangled CNTs were not cytotoxic to mesothelial cells and did not induce mesothelioma development. Mesothelial cell death results in the release of HMGB1. In humans, HMGB1 release induced by asbestos drives mesothelial cell transformation and the development of mesothelioma [14]. Thus, CNTs that are not cytotoxic to mesothelial cells are not carcinogenic in the pleural cavity, but these CNTs will interact with alveolar macrophages and consequently can be carcinogenic in the lung.

Two Non-carcinogenic CNTs

In another recent study we are investigating two types of CNTs that did not induce tumorigenesis after 2 years, NC-1 and NC-2. RNA sequence analysis of lung tissues collected 6 weeks after administration of NC-1 indicted that there was a foreign-body reaction in the lung, however, the overall changes in RNA expression induced by NC-1 was very different from that induced by carcinogenic-positive CNTs. Analysis of RNA expression indicted that inflammatory signaling induced by NC-1 was was much lower than that induced by carcinogenic-positive CNTs [Data presented at the PTCRF International Symposium,

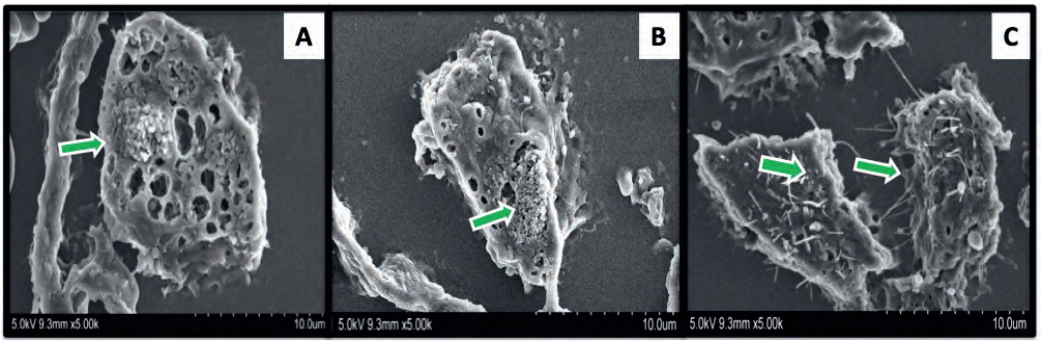

Figure 2 SEM examination of CNTs in the lung at 6 weeks. (A) NC-1. (B) NC-2. (C) MWCNT-7. Arrows indicate the engulfed CNTs.

manuscript in preparation]. The low inflammatory signaling was confirmed by quantitative real time PCR. In addition, at 6 weeks there were no free fibers in the alveoli, and at the end of the 2 year study period no fibers outside of granulation tissue were present in the lung. Thus, NC-1 and NC-1 interaction with macrophages induced little or no inflammatory signaling, and NC-1 and NC-2 did not completely impede the ability of macrophages to sequester these CNTs in granulation tissue or to physically transport them out of the alveoli and into the ciliated airways.

A notable observation was the difference in the appearance of macrophages interacting with NT-1 and NT-2 compared to macrophages interacting with MWCNT-7 at 6 weeks (Fig. 2). This is in agreement with the premise that interaction of CNTs with alveolar macrophages is a key component of the carcinogenic potential of CNTs in the lung.

References

1. Grosse Y, Loomis D, Guyton KZ, Lauby-Secretan B, El Ghissassi F, Bouvard V, et al. Carcinogenicity of fluoro-edenite, silicon carbide fibres and whiskers, and carbon nanotubes. Lancet Oncol. 2014;15 13:1427-8; doi: 10.1016/S1470-2045(14)71109-X. https://www.ncbi.nlm.nih.gov/pubmed/25499275.
2. Group IW. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans; volume 111-Some Nanomaterials and some Fibres. 2017.
3. Kasai T, Fukushima S. Exposure of Rats to Multi-Walled Carbon Nanotubes: Correlation of Inhalation Exposure to Lung Burden, Bronchoalveolar Lavage Fluid Findings, and Lung Morphology. Nanomaterials (Basel). 2023;13 18; doi: 10.3390/nano13182598. https://www.ncbi.nlm.nih.gov/pubmed/37764628.
4. Kasai T, Umeda Y, Ohnishi M, Mine T, Kondo H, Takeuchi T, et al. Lung carcinogenicity of inhaled multi-walled carbon nanotube in rats. Part Fibre Toxicol. 2016;13 1:53; doi:

10.1186/s12989-016-0164-2. https://www.ncbi.nlm.nih.gov/pubmed/27737701.

5. Tsuda H, Alexander DB. Development of Intratracheal Intrapulmonary Spraying (TIPS) administration as a feasible assay method for testing the toxicity and carcinogenic potential of multiwall carbon nanotubes. In Vivo Inhalation Toxicity Screening Methods for Manufactured Nanomaterials. 2019:145-63.
6. Suzui M, Futakuchi M, Fukamachi K, Numano T, Abdelgied M, Takahashi S, et al. Multiwalled carbon nanotubes intratracheally instilled into the rat lung induce development of pleural malignant mesothelioma and lung tumors. Cancer Sci. 2016;107 7:924-35; doi: 10.1111/cas.12954. https://www.ncbi.nlm.nih.gov/pubmed/27098557.
7. Saleh DM, Alexander WT, Numano T, Ahmed OHM, Gunasekaran S, Alexander DB, et al. Comparative carcinogenicity study of a thick, straight-type and a thin, tangled-type multi-walled carbon nanotube administered by intra-tracheal instillation in the rat. Part Fibre Toxicol. 2020;17 1:48; doi: 10.1186/s12989-020-00382-y. https://www.ncbi.nlm.nih.gov/pubmed/33054855.
8. Saleh DM, Luo S, Ahmed OHM, Alexander DB, Alexander WT, Gunasekaran S, et al. Assessment of the toxicity and carcinogenicity of double-walled carbon nanotubes in the rat lung after intratracheal instillation: a two-year study. Part Fibre Toxicol. 2022;19 1:30; doi: 10.1186/s12989-022-00469-8. https://www.ncbi.nlm.nih.gov/pubmed/35449069.
9. Hojo M, Maeno A, Sakamoto Y, Ohnuki A, Tada Y, Yamamoto Y, et al. Two-year intermittent exposure of a multiwalled carbon nanotube by intratracheal instillation induces lung tumors and pleural mesotheliomas in F344 rats. Part Fibre Toxicol. 2022;19 1:38; doi: 10.1186/s12989-022-00478-7. https://www.ncbi.nlm.nih.gov/pubmed/35590372.
10. Hojo M, Maeno A, Sakamoto Y, Yamamoto Y, Taquahashi Y, Hirose A, et al. Time-Course of Transcriptomic Change in the Lungs of F344 Rats Repeatedly Exposed to a Multiwalled Carbon Nanotube in a 2-Year Test. Nanomaterials (Basel). 2023;13 14; doi: 10.3390/nano13142105. https://www.ncbi.nlm.nih.gov/pubmed/37513116.
11. Nagai H, Okazaki Y, Chew SH, Misawa N, Yamashita Y, Akatsuka S, et al. Diameter and rigidity of multiwalled carbon nanotubes are critical factors in mesothelial injury and carcinogenesis. Proc Natl Acad Sci U S A. 2011;108 49:E1330-8; doi: 10.1073/pnas.1110013108. https://www.ncbi.nlm.nih.gov/pubmed/22084097.
12. Rittinghausen S, Hackbarth A, Creutzenberg O, Ernst H, Heinrich U, Leonhardt A, Schaudien D. The carcinogenic effect of various multi-walled carbon nanotubes (MWCNTs) after intraperitoneal injection in rats. Part Fibre Toxicol. 2014;11:59; doi: 10.1186/s12989-014-0059-z. https://www.ncbi.nlm.nih.gov/pubmed/25410479.
13. Sakamoto Y, Hojo M, Kosugi Y, Watanabe K, Hirose A, Inomata A, et al. Comparative study for carcinogenicity of 7 different multi-wall carbon nanotubes with different physicochemical characteristics by a single intraperitoneal injection in male Fischer 344

rats. J Toxicol Sci. 2018;43 10:587-600; doi: 10.2131/jts.43.587. https://www.ncbi.nlm.nih.gov/pubmed/30298847.

14. Xue J, Patergnani S, Giorgi C, Suarez J, Goto K, Bononi A, et al. Asbestos induces mesothelial cell transformation via HMGB1-driven autophagy. Proc Natl Acad Sci U S A. 2020;117 41:25543-52; doi: 10.1073/pnas.2007622117. https://www.ncbi.nlm.nih.gov/pubmed/32999071.

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COLLABORATIVE CROSS MICE: A POPULATION-BASED MODEL FOR OPTIMALLY DECIPHERING GENE-ENVIRONMENT-MICROBIOME INTERACTIONS IN CARCINOGENESIS

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The development of most cancers is caused by the complex interplay between genetic and environmental factors. The advent of biotechnologies, especially next generation sequencing technologies, has allowed the rapid discovery of genetic variants that contribute to cancer risk. Hundreds of cancer risk genes have been identified, but only a small proportion of cancer risk is explained by the genetic variants identified so far, known as "missing heritability". It has been shown that gene-environment interactions can provide valuable insights into missing heritability. Indeed, recent studies provide evidence that additional genetic and environmental factors can be identified in studies that examine gene-environment interactions.

We have recently realized that microbiomes play important roles in cancer development and progression. It has been reported that the microbiome is involved in the vast majority of cancer hallmarks such as genomics instability and mutation, deregulating cellular energetics, avoiding immune destruction, inducing angiogenesis, sustaining proliferative signal, tumor-promoting inflammation, and activating invasion and metastasis. The microbiome has now been suggested as a third factor that can affect cancer risk via its interaction with genetics and environment. Incorporating the contribution of the microbiome to cancer risk will lead to a more complete understanding of cancer and may shed light on strategies to halt or mitigate those contributions. Moreover, we have increased awareness of the impact of bidirectional interactions between genetics and microbiota and between environmental factors and microbiota on cancer risk. Understanding the causal role of the microbiome in this complex interaction is essential for the development of microbiome-targeted therapy for the prevention and treatment of cancer.

Gene-environment-microbiome interactions in cancer risk: It is widely accepted that gene-environment interactions influence cancer risk. Environmental exposures can cause genetic mutations, alter gene expression, etc., whereas interindividual variation in genetic makeup strongly determines the effect of environmental exposure on cancer risk. Consequently, investigations on gene–environment interactions are crucial in understanding how genetic heterogeneity is influenced by various environmental factors, getting more insights into the biological nature of cancer.

In recent years, attention has been raised to bidirectional interactions between host genetics and microbiome. Increasing number of studies have shown that host genetics is one of factors that determine the human microbiome composition and diversity. The genome wide association studies (GWAS) have identified hundreds of genetic variants significantly correlated to the abundance of distinct gut microbes. On the other hand, the microbiome strongly influences the host genome. Mounting evidence has shown that gut microbiota modulates host gene expression through dysregulation of the epigenetic landscape, direct interruption of host signaling cascades, chromatin remodeling, splicing alteration, and miRNAs. In addition, gut microbiota can produce genotoxic metabolites that cause DNA damage, subsequently leading to genomics instability, chromosomal aberrations, and gene mutations. Therefore, the bidirectional interactions between genetics and microbiome play a vital role in cancer risk.

Scientific research has begun to elucidate the various ways in which the human microbiome interacts with environmental exposures. The gut microbiota is a pivotal player in the toxicity of environmental pollutants, whereas environmental exposures are a crucial factor for human microbial composition and function. The human microbiome can metabolize environmental chemicals, either increasing or decreasing their toxicity to the mammalian host. Although the roles of microbiomes in the metabolism of environmental pollutants are established, there are substantial gaps in our understanding of the breadth of potential metabolic pathways of environmental pollutants in each microbiome. Alternately, environmental pollutants can, directly and indirectly, alter the microbiome. These changes in the microbiome by environmental pollutants can be detected by multi-omics. However, how environmental pollutants induce gut microbiota dysbiosis and its influence on cancer risk need to be investigated in future studies. The collective findings indicate that the bidirectional interactions between environmental factors and microbiome play an important contribution to cancer risk. Overall, we should consider genetic variants, environmental factors, and microbiome as multiplicative/additive factors in the prediction of cancer risk.

Collaborative Cross is an optimal mouse model for deciphering gene-environment-microbiome interactions in carcinogenesis: Many challenges have been faced in performing

human studies to decipher gene-environment-microbiome interactions in carcinogenesis, which partly originate from the complex, evolving, and expanding nature of genetic, environmental, and microbial data collection. Mouse models have contributed greatly to our current understanding of gene-environment interactions in cancer development and progression. More importantly, the mouse research community has established new population-based resources, such as the Collaborative Cross (CC) mouse resource.

The CC is a large panel of new inbred mouse strains derived from a genetically diverse set of eight inbred founder strains (A/J, C57BL/6J, 129S1/SvImJ, NOD/LtJ, NZO/HlLtJ, CAST/EiJ, PWK/PhJ, and WSB/EiJ). A targeted funneling breeding approach was used to randomize the genetic makeup of each inbred line. The CC collection allows studies to mimic the genetic diversity found in the human population and captures nearly 90% of the known variation of laboratory mice [1]. This system is a new innovative resource for studying systems genetics, allowing studies to rapidly map genetic loci at high resolution and identify individual genes involved in complex diseases at unprecedented speed. An advantage to using this system is that the genotype of each individual strain is already known, and since each CC line has been inbred, one can make repeated measurements in the same genetically diverse series of mice. Our studies in CC mice have reported the diversity in gut microbiome composition and metabolome [2, 3]; spontaneous tumor development [4]; cancer susceptibility to thirdhand smoking exposure [5]; blood immune response to radiation exposure [6]; and azoxymethane-induced acute toxicity [7]. In our recent study, we identified genetic and microbial determinants of azoxymethane-induced colorectal tumor susceptibility with human translational value. Moreover, we showed critical importance of interaction between genetics and microbiome in colorectal tumor susceptibility, which provides potential novel targets for personalized colorectal cancer prevention and treatment. Overall, all these studies demonstrated large variation in tumor susceptibility and tumor-related phenotypes across CC mouse strains, which suggests that CC mice phenotypically mimic the human populations. Therefore, CC mice are an optimal model for deciphering gene-environment-microbiome interactions in carcinogenesis, in combination with other mouse model such as knockout and gnotobiotic mice, which will provide the information about the causative mechanisms.

In conclusion, understanding gene-environment-microbiome interactions provides crucial insights into biological mechanisms and strategies for cancer prevention and control.

References

1. Collaborative Cross C: The genome architecture of the Collaborative Cross mouse genetic reference population. *Genetics* 2012, 190:389-401.

2. Snijders AM, Langley SA, Kim YM, Brislawn CJ, Noecker C, Zink EM, Fansler SJ, Casey CP, Miller DR, Huang H, Karpen GH, Celniker SE, Brown JB, Borenstein E, Jansson JK, Metz TO, Mao JH. Influence of early life exposure, host genetics and diet on the mouse gut microbiome and metabolome. *Nat Microbiol*, 2: 16221 (2017).
3. Mao JH, Kim YM, Zhou YX, Hu D, Zhong C, Chang H, Brislawn C, Langley S, Wang Y, Loulou Peisl BY, Celniker SE, Threadgill DW, Wilmes P, Orr G, Metz TO, Jansson JK, Snijders AM. Genetic and metabolic links between the murine microbiome and memory. *Microbiome*, 8: 53 (2020).
4. Wang P, Wang Y, Langley SA, Zhou YX, Jen KY, Sun Q, Brislawn CJ, Rojas CM, Wahl KL, Wang T, Fan X, Jansson JK, Celniker SE, Zou X, Threadgill DW, Snijders AM, Mao JH. Diverse tumor susceptibility in Collaborative Cross mice: Identification of a new mouse model for human gastric tumorigenesis. *Gut*, 68: 1942-1952 (2019).
5. Yang H, Wang X, Wang P, He L, Schick SF, Jacob P, Benowitz N, Gundel LA, Zhu C, Xia Y, Inman JL, Chang H, Snijders AM, Mao JH, Hang B. Thirdhand tobacco smoke exposure increases the genetic background-dependent risk of pan tumor development in Collaborative Cross mice. *Environ Int*, 174: 107876 (2023).
6. He L, Zhong C, Chang H, Inman JL, Celniker SE, Ioakeim-Ioannidou M, Liu KX, Haas-Kogan D, MacDonald SM, Threadgill DW, Kogan SC, Mao JH, Snijders AM. Genetic architecture of the acute and persistent immune cell response after radiation exposure. *Cell Genomics*, 3: 100422 (2023).
7. Zhong C, He L, Lee SY, Chang H, Zhang Y, Threadgill DW, Yuan Y, Zhou F, Celniker SE, Xia Y, Snijders AM, Mao JH. Host genetics and gut microbiota cooperatively contribute to azoxymethane-induced acute toxicity in Collaborative Cross mice. Arch Toxicol, 95: 949-958 (2021).

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THE HUMAN MICROBIOME AND CANCER PREVENTION

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My research focuses on how the social environment influences the microbiome and cancer prevention. My recent contributions to cancer prevention fall into three main categories:

1. Microbiome and cancer incidence: In our seminal papers relating periodontal disease to the risk of orodigestive track cancer[1], we tested the hypothesis that oral microbiome dysbiosis is related to orodigestive track cancer. In a large cohort study, we demonstrated that the oral carriage of commensal beta-proteobacteria predicts the subsequent development of head and neck cancer[2] and esophageal cancer[3]. Subsequently, we found that several pathogenic periodontal bacteria are associated with an increased risk of pancreatic cancer[4]. Mechanistic experiments revealed that cancer-associated oral microbiota play a pivotal role in metabolizing carcinogens in the oral cavity, and their abundance is significantly altered in heavy smokers and alcohol drinkers[5]. These studies were the first to establish the link between oral microbiota and orodigestive track cancer development. The impact on the field is further demonstrated by our recent invitation to contribute to the *2022 US Surgeon General's Report on Oral Health*.

2. Microbiome and cancer treatment response: We also demonstrated that the microbiome is a predictor of therapy response in cancer patients. My team also showed, in non-small cell lung cancer patients, that microbiome and peripheral blood gene expression biomarkers may improve risk prediction of recurrence in early-stage non-small cell lung cancer patients[6]. We demonstrated, in melanoma patients, that the gut microbiome also is a potential predictor for recurrence[7] and toxicity[8] after immune checkpoint inhibitor treatment. Bringing the

microbiome discovery to clinical utilization, we recently developed risk prediction models for immunotherapy decision-making in a large global immunotherapy trial (NYU Melanoma SPORE project).

3. Sociobiome and cancer health disparities: As the microbiome is influenced by the physical and socioeconomic environment[9], I developed FAMiLI, the only Asian American-enriched multi-ethnic cohort of 14,000 individuals, uniquely capturing microbiome, environmental, acculturation, and sociocultural factors in pre- and post-immigration status. This is particularly significant, as over 90% of Asian Americans in the U.S. are first-generation or second-generation immigrants. In this cohort, we found that the socioeconomic neighborhood environment[10] and dietary acculturation[11] influence gut microbiota associated with colon carcinogenesis. This cohort also forms the foundation of the NYU Cancer Health Disparities SPORE, which she serves as PI. As part of the SPORE initiative, our team is testing the novel hypothesis that the human microbiome contributes to racial and social disparities in oro-digestive track cancers.

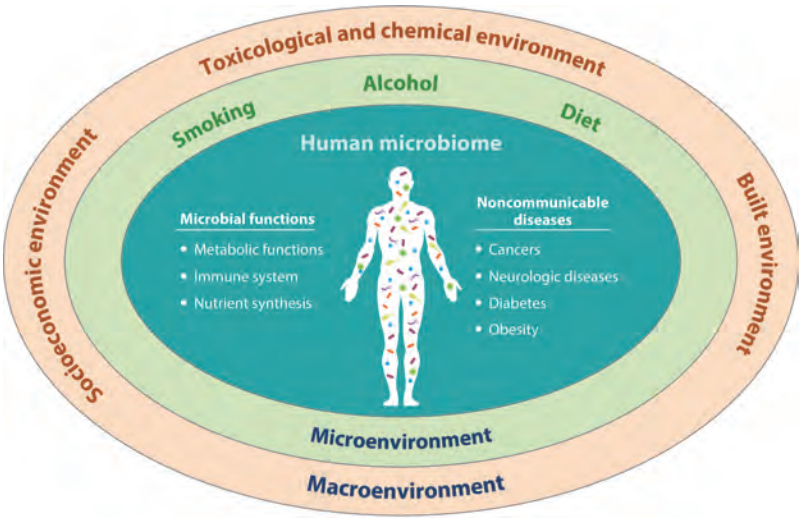

Figure 1 The effect of the environment on cancer is a complex set of interactions between multiple exposures that, alone or more commonly interdependently, affect various structures and functions of the microbiome. This figure illustrates how environmental exposures have a direct impact on the human microbiome, implicated in cancer. These environmental exposures are influenced by and interrelated with the macroenvironment, including built environment, and social environment, as well as the microenvironment, including smoking, alcohol, and dietary factors. Although external environmental impacts are illustrated, individual factors, such as age, sex, and genes, also interact with and eventually determine exposure, dose, and any subsequent response and effect. Ahn et al, Annual Review of Public Health, 2021

References

1. Ahn J, Segars S, Hayes RB. Periodontal Disease, Porphyromonas Gingivalis (P. gingivalis) Serum Antibody Levels and Orodigestive Track Cancer Mortality. Carcinogenesis. 2012;33(5):1055-8.
2. Hayes RB, Ahn J, Fan X, Peters BA, Wu J, Yang L, Purdue MP, Freedman ND, Gapstur SM, Pei Z. Oral microbiome and prospective risk for head and neck cancer. JAMA oncology. 2018;doi: 10.1001/jamaoncol.2017.4777. [Epub ahead of print].
3. Peters BA, Wu J, Pei Z, Yang L, Purdue MP, Freedman ND, Jacobs EJ, Gapstur SM, Hayes RB, Ahn J. Oral Microbiome Composition Reflects Prospective Risk for Esophageal Cancers. Cancer Research. 2017;77(23):6777-6787. doi: 10.1158/0008-5472.CAN-17-1296.
4. Fan X, Alekseyenko V, Jacobs E, Wu J, Peters B, Purdue MP, Miller G, Abnet CC, Hayes RB, Ahn J. Human oral microbiota and prospective risk for pancreatic cancer: a population-based nested case-control study. Gut 2018;67(1):120-127. doi: 10.1136/gutjnl-2016-312580. Epub 2019 Oct 14.
5. Wu J, Peters B, Dominianni C, Zhang Y, Pei Z, Yang L, Ma Y, Purdue MP, Jacobs EJ, Gapstur SM, Li H, Alekseyenko AV, Hayes RB, Ahn J. Cigarette smoking and the oral microbiome in a large study of American adults. ISME Journal. 2016(10):2432-46.
6. Peters BA, Pass HI, Burk RD, Xue X, Goparaju C, Sollecito CC, Grassi E, Segal LN, Tsay JJ, Hayes RB, Ahn J. The lung microbiome, peripheral gene expression, and recurrence-free survival after resection of stage II non-small cell lung cancer. Genome medicine. 2022;14(1):121. Epub 20221027. doi: 10.1186/s13073-022-01126-7. PubMed PMID: 36303210; PMCID: PMC9609265.
7. Peters BA, Wilson M, Moran U, Pavlick A, Izsak A, Wechter T, Weber JS, Osman I, Ahn J. Relating the gut metagenome and metatranscriptome to immunotherapy responses in melanoma patients. Genome medicine. 2019;11(1):61. Epub 20191009. doi: 10.1186/s13073-019-0672-4. PubMed PMID: 31597568; PMCID: PMC6785875.
8. Usyk M, Pandey A, Hayes RB, Moran U, Pavlick A, Osman I, Weber JS, Ahn J. Bacteroides vulgatus and Bacteroides dorei predict immune-related adverse events in immune checkpoint blockade treatment of metastatic melanoma. Genome medicine. 2021;13(1):160. Epub 20211013. doi: 10.1186/s13073-021-00974-z. PubMed PMID: 34641962; PMCID: PMC8513370.
9. Ahn J, Hayes RB. Environmental Influences on the Human Microbiome and Implications for Noncommunicable Disease. Annual review of public health. 2021;42:277-92. doi: doi: 10.1146/annurev-publhealth-012420-105020; PMCID: PMC8641399.
10. Ahn J, Kwak S, Usyk M, Beggs D, Choi H, Ahdoot D, Wu F, Maceda L, Li H, Im EO, Han HR, Lee E, Wu A, Hayes R. Sociobiome - Individual and neighborhood socioeconomic status influence the gut microbiome in a multi-ethnic population in the US. Res Sq. 2023.

Epub 20230419. doi: 10.21203/rs.3.rs-2733916/v1. PubMed PMID: 37131763; PMCID: PMC10153375.

11. Peters BA, Yi SS, Beasley JM, Cobbs EN, Choi HS, Beggs DB, Hayes RB, Ahn J. US nativity and dietary acculturation impact the gut microbiome in a diverse US population. ISME J. 2020;14(7):1639-50. Epub 20200324. doi: 10.1038/s41396-020-0630-6. PubMed PMID: 32210364; PMCID: PMC7305110.

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HISTONE H3.3-SPECIFIC CHAPERONE HIRA IS REQUIRED FOR ACQUIRED TOLERANCE, A FORM OF PHENOTYPIC PLASTICITY

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It remains an enigma as to why cancer cells, once they reach an advanced stage, continue to exhibit robust growth and metastasis despite repeated treatments. Recent studies focus on the potential implication of non-genetic changes of phenotypes, specifically the concept of "phenotypic plasticity", in the context of cancer progression. Hormesis collectively designates phenomena wherein low-dose stresses bestow pro-survival effects on cells and organisms, as opposed to anti-survival effects induced by high-dose stresses. Acquired tolerance (AT) is a form of hormesis, wherein pretreatment of cells with low-dose stress confers resistance to subsequent lethal stresses. It has long been recognized that mild ischemia induces resistance to subsequent profound ischemia in the heart and brain (also known as post-conditioning tolerance). The anti-aging effect of caloric restriction is arguably a hormetic effect brought about by mild starvation. They are possibly examples of AT.

Although AT has generated substantial attention to explain various physiological and pathological conditions, its molecular mechanisms remained largely unknown. To address the question, we first devised experimental systems to study AT in fission yeast. Classical forward genetics identified histone chaperone HIRA is required for AT in fission yeast[1].

In this study, we have extended our analyses to humans and elucidated the molecular mechanism in detail[2]. Using normal human fibroblast WI-38 cells and cancer cell lines, we have established low-dose heat stress (referred to as priming, P) elicited AT towards subsequent lethal heat stress (L). When we knock-downed the *HIRA* gene encoding a subunit of the mammalian HIRA trimeric complex, we found that AT was significantly inactivated. We therefore concluded that HIRA plays a pivotal role in AT across species, from fission yeast to humans.

HIRA functions as a histone H3.3-specific chaperone. In contrast to the conventional histone H3, H3.1 and H3.2, H3.3 is synthesized throughout the cell cycle and in non-proliferating cells. It is substituted for H3.1 and H3.3, and activate genes. Employing ChiP-seq, we demonstrated that priming heat stress loaded histone H3.3 to stress-responsive genes, including those encoding heat shock proteins (HSPs). RNA-seq experiments consistently revealed that HSPs are profoundly up-regulated by priming + lethal (PL) conditions compared to P alone or L alone.

We furthermore conducted the DMBA-TPA-induced skin carcinogenesis in *HIRA*$^{-/-}$ and control mice. Interestingly, tumors in KO mice showed significantly slow progression and frequent spontaneous regression of tumors relative to control mice. We conclude that HIRA-H3.3 axis constitutes a promising target to prevent cancer progression.

This research was conducted through a collaborative effort with Drs. Ryoji Yao (Japanese Foundation for Cancer Research), Yuichi Wakabayashi (Chiba Cancer Center Research Institute), Ryuichiro Nakato, and Katsuhiko Shirahige (Institute for Quantitative Biosciences, The University of Tokyo). It was supported by Grants from The Japan Agency for Medical Research and Development (AMED), MEXT KAKENHI Grant and Princess Takamatsu Cancer Research Fund.

References

1. Chujo, M., Tarumoto, Y., Miyatake, K., Nishida, E., and Ishikawa, F. (2012). HIRA, a conserved histone chaperone plays an essential role in low-dose stress response via transcriptional stimulation in fission yeast. ***J Biol Chem*** 287(28), 23440-23450. doi: 10.1074/jbc.M112.349944.
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EPIGENETICS AND ENVIRONMENTAL ORIGINS OF CANCER

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Epigenetics, traditionally defined as stable and heritable changes impacting gene expression that are independent of changes on DNA sequence, has become a mainstrem in cancer research owing to the recognition that epigenetic mechanisms are the key contributors to cancer development and progression. Epigenome changes, with their ubiquitous presence and great potential for modulation, have become a major focus in mechanistic studies and biomarker discovery, and in epigenetics-based cancer therapy and prevention. A plethora of recent studies has also highlighted the important role of epigenome deregulation in mediating gene–environment interactions and their effects throughout the tumorigenesis process. This is supported by growing evidence showing that epigenetic changes may be risk factor-specific ("fingerprints"), which should prove instrumental in the discovery of new biomarkers in cancer [1]. This progress has been catalysed by advances in epigenomics, including the emergence of powerful technologies and state-of-the-art *in vitro* as well as *in silico* computational approaches. It is noteworthy that well-established risk factors of cancer, such as smoking, alcohol, fungal toxins, biological agents, diet and age, as well as air pollution and endocrine disruptors, have been shown to act though epigenome deregulation [2]. In recent years, many international cohorts and consortia have been established, which together with sequencing technologies have galvanized investigations of epigenetic precursors of cancer and biomarkers of risk in the context of large-scale molecular epidemiology studies, including those aimed at functional characterization of the mechanistically important (driver) genes [3]. The intrinsic reversibility of epigenetic alterations raises the prospect of the development of novel therapeutic and preventive strategies.

Epigenetic signatures of early-life factors and childhood cancer risk

Growing evidence points to an origin *in utero* of pediatric cancers, when global redistribution of the epigenome modifications occurs driving tissue differentiation [1]. Recent international efforts are aimed testing the hypothesis that molecular causes of cancers in children and adolescents innvolve epigenetic changes driven by risk factors exposures. We and other put forward the hypothesis that epigenetic changes associated with exposure to environmental/lifestyle factors during pregnancy and disparity drivers can be identified in blood cells at birth, and that these changes can serve as sensitive biomarkers in primary and secondary prevention of pediatric cancer. Such studies build on recent advances in identifying epigenetic markers of early-life factors and deciphering their precursor roles in pediatric cancer and its predisposing (intermediate) phenotypes at critical ages of development, exploiting large international networks of prospective and retrospective consortia some of which are coordinated by the International Agency for Research on Cancer (IARC, epichildcan.iarc.who.int, [4-6]). These include: (i) extending the exposure period from fetal life to childhood and adolescence by bringing together new consortia, lager sample sizes, and previously uncharacterized geographical regions, notably different ethnicities and disparity populations (including low- and middle-income countries, LMICs), and (ii) innovative study designs and approaches (particularly those that can overcome statistical power limitations associated with rare diseases such as pediatric cancer). These international efforts should improve our knowledge of the etiology of pediatric cancer and identify both novel biomarkers and clues to causation, thus providing an evidence base for cancer prevention and strategies aimed at reducing disparities. The ability to monitor exposure to cancer-promoting factors and screen for early changes that suggest increased cancer risk will have an important impact on the design and follow-up of preventive measures.

Identifying epigenetic driver genes ('epidrivers') in cancer and their link to environmental carcinogens

Recent large-scale international sequencing studies of the human cancer genome, spearheaded by The Cancer Genome Atlas (TCGA), revealed the high frequency of mutations in epigenetic regulator genes (ERGs) in common human cancers. We hypothesized that these genes may act as "drivers" ("epidrivers") of tumorigenesis and that they constitute critical mechanisms promoting epigenome changes that are evident in human malignancies and potentially synergize with environmental exposure.

We conducted a pan-cancer and comprehensive characterization of genomic and transcriptomic alterations of ERGs by applying a battery of bioinformatics, biostatistics, and experimental tools [3, 7]. Our analysis integrated (epi)genome, transcriptome, and DNA methylome alterations in a comprehensive compendium of 426 ERGs across 33 cancer types,

comprising >11,000 tumor and normal tissues. We also developed and applied a conceptual framework (including an orthogonal in vitro screen CRISPR-Cas9 screen targeting all ERGs) for experimental identification and characterization of the mechanistically important epidrivers that reshape the epigenome associated with environmental exposure.

We found that, in addition to mutations, copy number alterations (CNAs) in ERGs were more frequent than previously anticipated. A subset of ERGs was frequently deregulated at the genetic and/or RNA expression level across many malignancies, consistent with the notion that disruption of some ERGs represents a shared driver mechanism operating across multiple cancer types. By applying novel bioinformatics and statistical approaches, integrating the strengths of various driver prediction algorithms, we identified ERGs with potential driver roles within and across malignancies. We further orthogonally validated in cellular models functionally important epidriver genes through CRISPR/Cas9 screen by identifying a panel of ERGs with a potential epidriver role conferring on cancer cells the trait associated with specific hallmarks of cancer [3].

Our ongoing studies are aimed at investigating whether specific epidrivers have a central role in associating environmental exposures with cancer, including multiple phenotypic traits (and "epigenetic signatures") across the carcinogenesis process and using different biological systems. The observed deregulations in ERGs represent snapshot portraits of accumulated molecular events (mutations and non-mutational alterations) captured at a given (often advanced) time point of a multistep process; thus, the timing and role of critical epidriver events at critical stages in cancer are poorly understood [8]. Therefore, epidriver-based disruption of cellular processes may not only assume a primary role at different stages of tumorigenesis but also dictate cancer outcome and phenotype. Interestingly, epigenomic (chromatin) organization has been proposed as a major determinant not only of gene expression programmes but also of the cancer mutational landscape. In line with this, chromatin accessibility and modification patterns were shown to explain a vast majority of the variance in mutation rates across cancer genomes, and that epigenomic features are best predictors of local somatic mutation density. Our ongoing studies are aimed at testing the hypothesis that changes in the epigenomic patterns as a consequence of epidriver deregulation may not only contribute to changes in the transcriptional programme but also acting as an important non-mutagenic mechanism of cancer promotion (Figure 1). These studies should make a contribution to understanding the causes of cancer by providing insights into the mechanisms by which environmental factors promote carcinogenesis, as well as powerful targets for personalized approaches in early cancer 'interception' strategies.

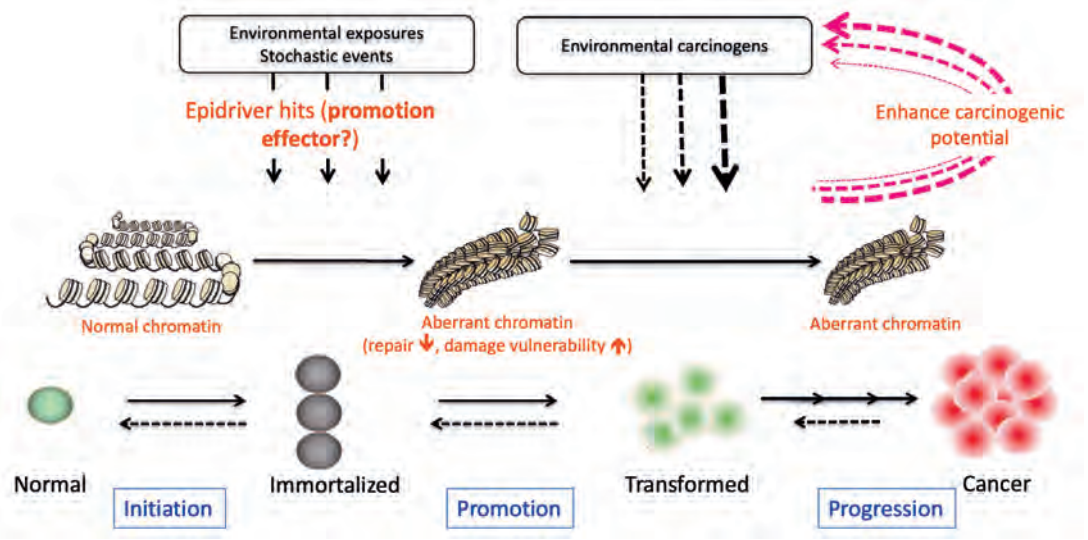

Figure 1 Hypothetical model of "epidrivers" enhancing/promoting potential of carcinogens. Epidriver hits occurring in cells (as results of environmental exposure or stochastic events, such as those associated with ageing) might trigger or promote the carcinogenic effect of exposure to environmental carcinogens, leading to the development and progression of cancer.

References

1. Herceg Z, Ghantous A, Wild CP, Sklias A, Casati L, Duthie SJ, Fry R, Issa JP, Kellermayer R, Koturbash I, et al: **Roadmap for investigating epigenome deregulation and environmental origins of cancer**. *Int J Cancer* 2018, **142**:874-882.
2. Chung F, Herceg Z: **The promises and challenges of toxico-epigenomics: Environmental chemicals and their impacts on the epigenome**. *Env Health Perspect* 2019, **in revision**.
3. Halaburkova A, Cahais V, Novoloaca A, Araujo M, Khoueiry R, Ghantous A, Herceg Z: **Pan-cancer multi-omics analysis and orthogonal experimental assessment of epigenetic driver genes**. *Genome Res* 2020, **30**:1517-1532.
4. Joubert BR, Felix JF, Yousefi P, Bakulski KM, Just AC, Breton C, Reese SE, Markunas CA, Richmond RC, Xu CJ, et al: **DNA Methylation in Newborns and Maternal Smoking in Pregnancy: Genome-wide Consortium Meta-analysis**. *Am J Hum Genet* 2016, **98**:680-696.
5. Kupers LK, Monnereau C, Sharp GC, Yousefi P, Salas LA, Ghantous A, Page CM, Reese SE, Wilcox AJ, Czamara D, et al: **Meta-analysis of epigenome-wide association studies in neonates reveals widespread differential DNA methylation associated with birthweight**. *Nat Commun* 2019, **10**:1893.
6. Merid SK, Novoloaca A, Sharp GC, Kupers LK, Kho AT, Roy R, Gao L, Annesi-Maesano I,

Jain P, Plusquin M, et al: **Epigenome-wide meta-analysis of blood DNA methylation in newborns and children identifies numerous loci related to gestational age**. *Genome Med* 2020, **12**:25.

7. Vicente A, Novoloaca A, Cahais V, Awada Z, Cuenin C, Spitz N, Carvalho AL, Evangelista AF, Crovador CS, Reis RM, et al: **Cutaneous and acral melanoma cross-OMICs reveals prognostic cancer drivers associated with pathobiology and ultraviolet exposure**. *Nat Commun* 2022, **13**:4115.
8. Feinberg AP, Koldobskiy MA, Gondor A: **Epigenetic modulators, modifiers and mediators in cancer aetiology and progression**. *Nat Rev Genet* 2016, **17**:284-299.

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EPIGENETIC FIELD FOR CANCERIZATION DUE TO CHRONIC INFLAMMATION

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Impact of chronic inflammation on normal-appearing epithelial cells

Chronic inflammation is induced by multiple environmental factors, including bacterial and viral infection, mechanical irritation, and chemical irritation, and can cause cancers. It is known that inflammation-associated cancers are heavily influenced by aberrant DNA methylation, such as gastric cancer induced by *Helicobacter pylori* (HP) infection-triggered inflammation [1]. Aberrant DNA methylation is not only present in cancer cells but also in normal-appearing tissues, long before cancer development, involving numerous genes [2]. Total deviation from a normal cell can be defined as an epigenetic burden, which can be represented by the methylation level of a single marker gene [3]. Importantly, the methylation level of such a marker gene is correlated with gastric cancer risk, reflecting the presence of an epigenetic field for cancerization (Fig. 1) [1]. The impact of epigenetic alterations on gastric cancer risk was stronger than point mutations [4].

Induction mechanism of aberrant DNA methylation

Aberrant DNA methylation is induced by chronic inflammation, not by *HP* itself [5]. Comparison of multiple types of persistent inflammation, that caused by *HP* infection, high salt, and high concentration of alcohol, showed that only HP infection-triggered inflammation can induce severe aberrant DNA methylation, and indicated that IL-1β/TNF-α signaling and increased *Nos2* expression were important [6]. At the molecular level, simultaneous suppression of *TET* genes, due to activation of NF-κB signalling via IL-1β/TNF-α signaling, and exposure to nitric oxide, due to increased *NOS2* expression, was responsible for methylation induction (Fig. 2) [7]. It is noteworthy that many types of chronic

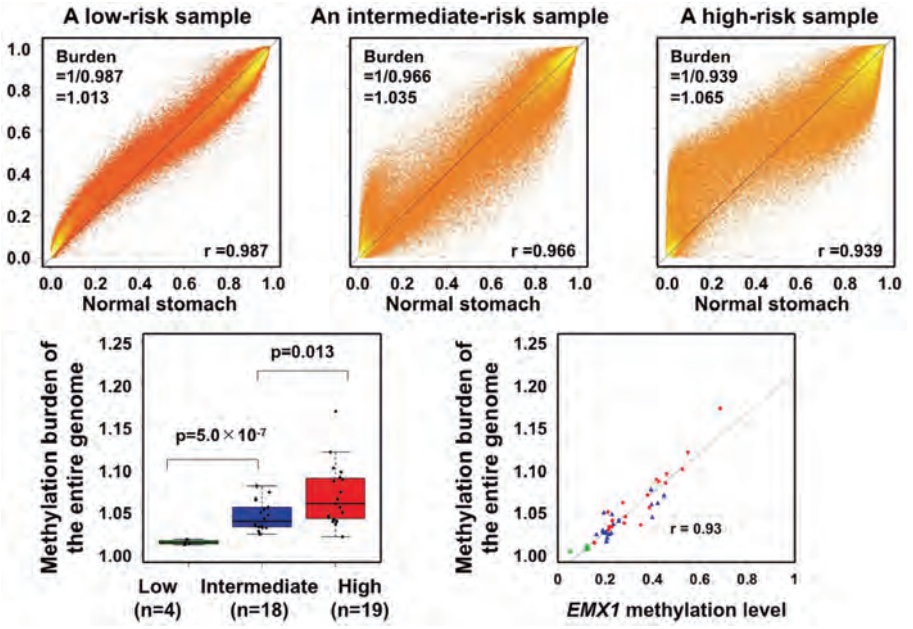

Fig. 1 Methylation burden in normal-appearing gastric mucosa.
Three representative samples were analyzed for their methylation deviation from normal stomach, and methylation burden was calculated as an inverse of a correlation coefficient. The methylation burden clearly increased according to risk levels, and was closely correlated with methylation level of a marker gene (modified from reference #3).

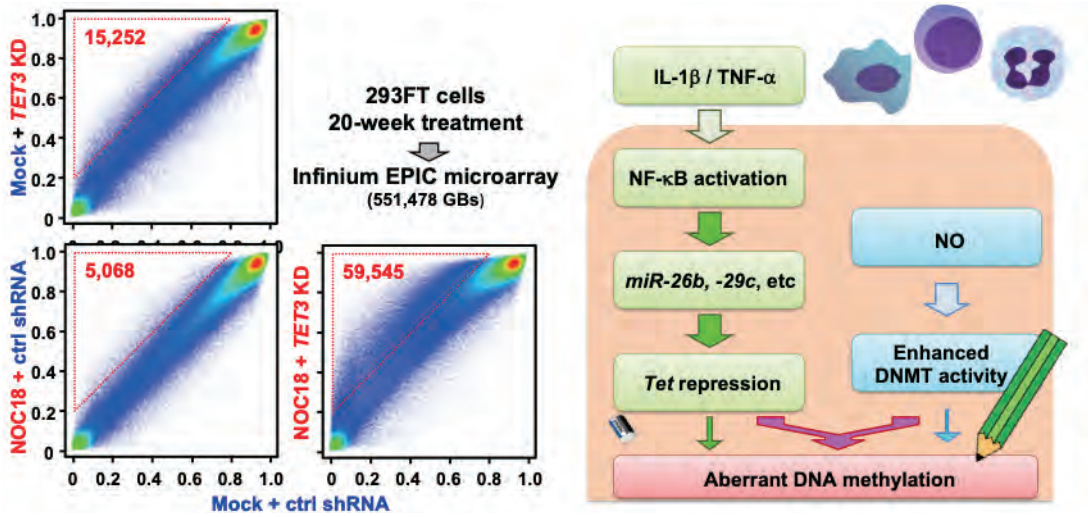

Fig. 2 Methylation induction by synergy of NF-κB activation and NO production.
Treatment of cells with *TET3*-targeting shRNA only induced mild aberrant DNA methylation (scatter plot, upper left), and that of cells with NO donor only induced minimal aberrant DNA methylation (scatter plot, bottom left). In contrast, simultaneous treatment of cells with *TET3*-targeting shRNA and NO donor induced extensive aberrant DNA methylation (scatter plot, right) (modified from reference #7).

inflammation associated with cancer are known to have high IL-1β/TNF-α and *NOS2* expression, and the same mechanism is likely to be involved.

Clinical application of aberrant DNA methylation in normal tissues

Based the fact that the accumulation level of aberrant DNA methylation is correlated with cancer risk and that chronic inflammation is mechanistically involved in its induction, clinical application of the epigenetic field as a gastric cancer risk marker is on-going. A multicenter prospective cohort study involving 826 gastric cancer patients cured by endoscopic treatment showed that risk of metachronous gastric cancer can be precisely predicted by the methylation level of a single marker gene, *miR-124a*, in gastric mucosa (Fig. 3) [8, 9]. Now, another multicenter prospective study involving 1,880 healthy people with severe atrophy after *HP* eradication has reached its target event number with a median follow-up period of 4 years, and its results will be disclosed soon.

Conclusion

Environmental exposure, especially chronic inflammation, induces aberrant DNA

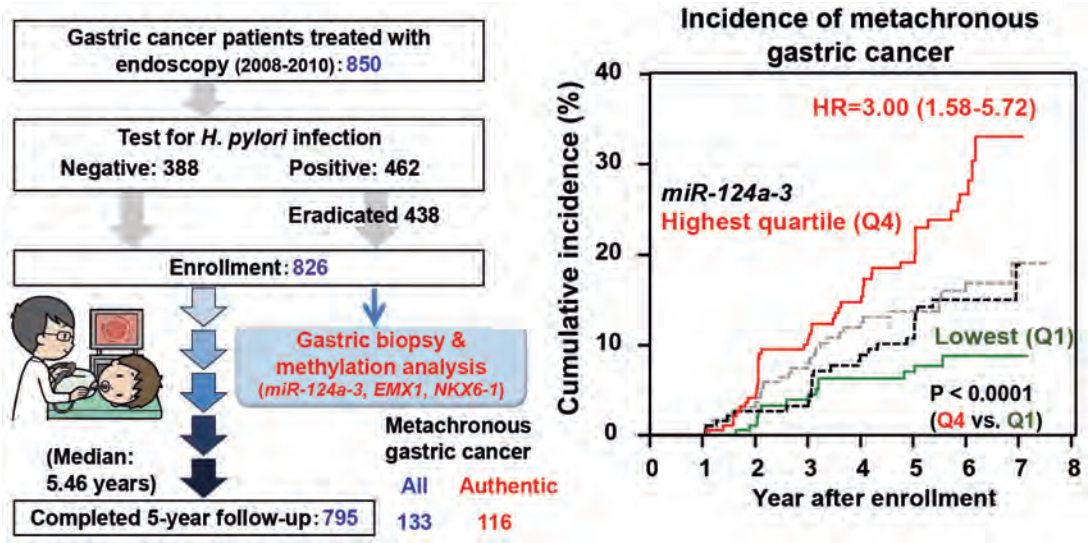

Fig. 3 Protocol and result of a multicenter prospective study to predict risk of metachronous gastric cancer after endoscopic treatment of initial gastric cancer.
A total of 850 gastric cancer patients cured for their initial gastric cancer were tested for *HP* infection, and, if positive, received eradication. Finally, 826 patients were enrolled, and one piece of biopsy was taken from their antrum. Methylation levels of three marker genes were measured, and they were followed up by annual endoscopy for five years. When patients were stratified by the methylation level of *miR-124a-3*, the highest quartile (Q4) had much higher risk of metachronous gastric cancer than the lowest quartile (Q1) with a hazard ratio of 3.0 (95%CI=1.58-5.72) (modified from references #8 and #9).

methylation of many genes in normal tissues, and this process is likely to be involved in a variety of human disorders.

References

1. Maekita, T, Nakazawa, K, Mihara, M, Nakajima, T, Yanaoka, K, Iguchi, M, Arii, K, Kaneda, A, Tsukamoto, T, Tatematsu, M, Tamura, G, Saito, D, Sugimura, T, Ichinose, M and Ushijima, T. High levels of aberrant DNA methylation in *Helicobacter pylori*-infected gastric mucosae and its possible association with gastric cancer risk. **Clin Cancer Res**, 12: 989-995, 2006.
2. Yamashita, S, Nanjo, S, Rehnberg, E, Iida, N, Takeshima, H, Ando, T, Maekita, T, Sugiyama, T and Ushijima, T. Distinct DNA methylation targets by aging and chronic inflammation: a pilot study using gastric mucosa infected with Helicobacter pylori. **Clin Epigenetics**, 11: 191, 2019.
3. Irie, T, Yamada, H, Takeuchi, C, Liu, YY, Charvat, H, Shimazu, T, Ando, T, Maekita, T, Abe, S, Takamaru, H, Kodama, M, Murakami, K, Sugimoto, K, Sakamoto, K and Ushijima, T. The methylation level of a single cancer risk marker gene reflects methylation burden in gastric mucosa. **Gastric Cancer**, 26: 667-676, 2023.
4. Yamashita, S, Kishino, T, Takahashi, T, Shimazu, T, Charvat, H, Kakugawa, Y, Nakajima, T, Lee, YC, Iida, N, Maeda, M, Hattori, N, Takeshima, H, Nagano, R, Oda, I, Tsugane, S, Wu, MS and Ushijima, T. Genetic and epigenetic alterations in normal tissues have differential impacts on cancer risk among tissues. **Proc Natl Acad Sci U S A**, 115: 1328-1333, 2018.
5. Niwa, T, Tsukamoto, T, Toyoda, T, Mori, A, Tanaka, H, Maekita, T, Ichinose, M, Tatematsu, M and Ushijima, T. Inflammatory processes triggered by *Helicobacter pylori* infection cause aberrant DNA methylation in gastric epithelial cells. **Cancer Res**, 70: 1430-1440, 2010.
6. Hur, K, Niwa, T, Toyoda, T, Tsukamoto, T, Tatematsu, M, Yang, HK and Ushijima, T. Insufficient role of cell proliferation in aberrant DNA methylation induction and involvement of specific types of inflammation. **Carcinogenesis**, 32: 35-41, 2011.
7. Takeshima, H, Niwa, T, Yamashita, S, Takamura-Enya, T, Iida, N, Wakabayashi, M, Nanjo, S, Abe, M, Sugiyama, T, Kim, YJ and Ushijima, T. TET repression and increased DNMT activity synergistically induce aberrant DNA methylation. **J Clin Invest**, 130: 5370-5379, 2020.
8. Asada, K, Nakajima, T, Shimazu, T, Yamamichi, N, Maekita, T, Yokoi, C, Oda, I, Ando, T, Yoshida, T, Nanjo, S, Fujishiro, M, Gotoda, T, Ichinose, M and Ushijima, T. Demonstration of the usefulness of epigenetic cancer risk prediction by a multicentre prospective cohort study. **Gut**, 64: 388-396, 2015.

9. Maeda, M, Nakajima, T, Oda, I, Shimazu, T, Yamamichi, N, Maekita, T, Asada, K, Yokoi, C, Ando, T, Yoshida, T, Nanjo, S, Fujishiro, M, Gotoda, T, Ichinose, M and Ushijima, T. High impact of methylation accumulation on metachronous gastric cancer: 5-year follow-up of a multicentre prospective cohort study. **Gut**, 66: 1721-1723, 2017.

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OBESITY, METABOLIC DYSFUNCTION AND CANCER: UNDERSTANDING CAUSAL MECHANISMS TO GUIDE PREVENTION

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Excess adiposity is a recognized risk factor for at least 13 cancer types including cancers of the colorectum, pancreas, liver, endometrium, ovary, kidney, gastric cardia, thyroid, gallbladder, breast (post-menopausal), bladder as well as oesophageal adenocarcinoma and leukaemia[1]. Global obesity rates continue to increase, and therefore the incidence of cancers attributable to excess adiposity are also likely to rise. In fact, it has been estimated that in the coming decades, obesity will surpass smoking as the leading cause of cancer in some countries[2]. Currently, 12% of adult males and 16% of adult females worldwide are classified as obese[3]. Among women, the highest prevalence of obesity is in Central Asia, the Middle East, and North Africa (age-standardised prevalence 35%). Among men, prevalence is highest in high income Western countries (30%)[3]. South Asia is experiencing the fastest relative increase in obesity rates, with prevalence increasing from 0.4% to 4.6% in recent decades. Obesity is also increasing in children having risen from 0.7% to 5.6% in girls and 0.9% to 7.8% in boys over the past 30-40 years[3].

Approximately 3.6% of all new cancer cases are estimated to be directly attributable to high body mass index (BMI) and this proportion is greater for women than for men (5.4% and 1.9%, respectively)[4]. High BMI is the third leading risk factor for the global cancer burden, behind tobacco and alcohol, accounting for 4.7 and 4.3% of disability-adjusted life years from cancer, for women and men, respectively[5]. This burden is highest in North America and has increased over time, but the fastest increases are occurring in low- and middle-income countries. Greater obesity prevalence in younger populations is also hypothesized to be contributing to rising cancer diagnoses at earlier ages[6].

Population-level strategies for reversing the obesity epidemic, even if successful, may be

insufficient for reducing cancer risk in many people with obesity, and effective, targeted prevention strategies are urgently needed. Critical questions, however, include whom to target and how? Tremendous metabolic and physiological heterogeneity occurs amongst people with obesity and only some develop cancer. Metabolic sub-states exist within the standard body mass index (BMI) sub-classes (e.g. defined by insulin levels)[7,8], and BMI does not capture variations in body composition, including fat and muscle distribution, metabolic dysregulation and inflammation/immune function, which may be highly relevant to cancer development. Biomarkers or well-characterised phenotypes that identify individuals at higher risk of cancer could guide precision interventions that intercept pro-cancer mechanisms.

Adipose tissue is a complex endocrine system that interacts with numerous organs to maintain metabolic homeostasis. Body fat levels correlate with circulating levels of hundreds to thousands of different metabolites, proteins, and other biomarkers[9-12]. It is possible that only a small fraction of these, e.g. 5% or fewer, are relevant to cancer risk. Moreover, only some individuals with obesity may have elevated levels of the cancer-pertinent factors. Approximately 10-30% of individuals with obesity maintain metabolic health characterised by an absence of conditions such as gallstones, diabetes, hyperinsulinemia, dyslipidaemia, or fatty liver[7,8]. These individuals may have lower cancer risks compared with their metabolically unhealthy counterparts, though risk remains higher than those who are metabolically healthy and lean.

Metabolic phenotypes, not always well-captured by BMI, are hypothesized to underlie the obesity-cancer link and promote the proliferation and survival of cells bearing oncogenic mutations. These 'metabotypes' likely have their origins in dysregulated body composition and adipose tissue; however, their underlying pro-cancer mechanisms are not understood. Knowledge of how obesity, body composition and weight change impact the biology of normal tissues at anatomical sites where obesity is associated with tumour development, is critical for understanding causal pathways and developing precise prevention interventions, targeted to mechanistic pathways. Human weight loss intervention studies using bariatric surgery, diet or pharmacological methods, with collection of tissues and biospecimens pre- and post-weight loss can provide novel insights into how weight loss in obesity can modify the molecular architecture of normal tissues[13,14]. In parallel, recent developments in epidemiological research, particularly the growing volume of health data and molecular measurements (e.g metabolomics, proteomics, imaging), has the potential to enhance our understanding of the biological pathways underlying the obesity-cancer link. Triangulation of such data with those from experimental models could uncover novel mechanisms linking obesity with cancer and ultimately may inform preventative strategies and formulation of effective public health policies.

References

1. Lauby-Secretan, B. et al. Body fatness and cancer. N. Engl. J. Med. 375, 794–798 (2016)
2. https://news.cancerresearchuk.org/2018/09/24/obesity-could-overtake-smoking-as-biggest-preventable-cause-of-cancer-in-women/
3. Abarca-Gómez, L. *et al.* Worldwide trends in body-mass index, underweight, overweight, and obesity from 1975 to 2016: a pooled analysis of 2416 population-based measurement studies in 128·9 million children, adolescents, and adults. *The Lancet* **390**, 2627–2642 (2017).
4. Arnold, M. *et al.* Global burden of cancer attributable to high body-mass index in 2012: a population-based study. *Lancet Oncol.* **16**, 36–46 (2015).
5. Tran, K. B. *et al.* The global burden of cancer attributable to risk factors, 2010–19: a systematic analysis for the Global Burden of Disease Study 2019. *The Lancet* **400**, 563–591 (2022).
6. Sung, H., Siegel, R. L., Rosenberg, P. S. & Jemal, A. Emerging cancer trends among young adults in the USA: analysis of a population-based cancer registry. *Lancet Public Health* **4**, e137–e147 (2019).
7. Murphy, N. et al. Nested case-control study of metabolically defined body size phenotypes and risk of colorectal cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC). PLOS Med. 13, e1001988 (2016)
8. Gunter MJ, et al. Breast cancer risk in metabolically healthy but overweight postmenopausal women. Cancer Res. 2015 Jan 15;75(2):270-4.
9. Moore, S. C. *et al.* A Metabolomics Analysis of Body Mass Index and Postmenopausal Breast Cancer Risk. *JNCI J. Natl. Cancer Inst.* (2018) doi:10.1093/jnci/djx244.
10. Cirulli, E. T. *et al.* Profound Perturbation of the Metabolome in Obesity Is Associated with Health Risk. *Cell Metab*. **29**, 488-500.e2 (2019).
11. Zaghlool, S. B. *et al*. Revealing the role of the human blood plasma proteome in obesity using genetic drivers. *Nat. Commun*. **12**, 1279 (2021).
12. Goudswaard, L. J. *et al*. Effects of adiposity on the human plasma proteome: observational and Mendelian randomisation estimates. *Int. J. Obes. 2005* **45**, 2221–2229 (2021).
13. Beeken RJ, et al. The Impact of Diet-Induced Weight Loss on Biomarkers for Colorectal Cancer: An Exploratory Study (INTERCEPT). Obesity 2017 Nov;25 Suppl 2:S95-S101
14. Naqvi A, et al. The impact of obesity and bariatric surgery on the immune microenvironment of the endometrium. Int J Obes (Lond). 2022 Mar;46(3):605-612.

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MOLECULAR PREVENTION OF CANCER WITH ASPIRIN

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Over the last several decades, research into cancer prevention has focused on early detection (e.g. screening) or unselected chemoprevention, defined as the use of agents (e.g. medications, vitamins, or supplements) for the inhibition, delay, or reversal of carcinogenesis. Although screening has had many notable successes, there are barriers to implementation and inherent limitations to its efficacy. For chemoprevention, the field has largely tested putative agents through expensive Phase III clinical trials in large populations of generally unselected individuals which has yielded few successes. A notable exception is the remarkably consistent evidence demonstrating that aspirin is associated with a lower risk of colorectal cancer (CRC), which led to a first-if-its-kind U.S. Preventive Services Task Force (USPSTF) recommendation for population use of aspirin for cancer prevention in 2016. The 2016 USPSTF recommendations were a milestone, endorsing for the first time low-dose aspirin for prevention of colorectal cancer in addition to cardiovascular disease (CVD) for individuals aged 50–59 years with a 10% ten-year cardiovascular risk [1].

A shift in guidelines: In 2022, the USPSTF released new guidelines reversing this recommendation based on an evidence synthesis that prioritized the results of new primary CVD prevention aspirin RCTs completed after 2016: ASCEND (A Study of Cardiovascular Events in Diabetes); ARRIVE (Aspirin to Reduce Risk of Initial Vascular Events); and ASPREE (Aspirin to Prevent Events in the Elderly) [2-4]. These trials did not show a lower risk of CRC. However, a crucial limitation of each of these trials is the short duration of follow-up (median 4.7 to 7.4 years), undoubtedly too brief for a protective benefit of aspirin

to emerge. Prior trials considered for the 2016 recommendations clearly demonstrated that reduction in CRC and CRC mortality is not evident until at least 10 years, consistent with what might be expected for a slowly evolving process such as cancer.

Limitations of ASPREE's duration of follow-up: The 2022 USPSTF also heavily weighted findings from ASPREE, which enrolled U.S minority participants of at least 65 years of age or Australian/U.S. White participants of at least 70 years of age. ASPREE showed low-dose aspirin increased CRC mortality (as well as mortality from cancer arising from other anatomic sites) after a median of 4.7 years, a sharp contrast with prior trial data [5]. However, ASPREE did not prespecify CRC mortality as an endpoint, included only a small number of events, and did not adjust its analysis for multiple comparisons (18 different cancer endpoints). The trial was also stopped prematurely due to futility for the primary endpoint (disability and dementia-free survival), increasing the likelihood of artefactual results. Thus, the USPSTF's focus on the outlier finding of increased CRC mortality in ASPREE as a potential signal of broader harm seems an overreach. In a subsequent study examining incident cancer events rather than cancer deaths, we worked with the ASPREE team to show that aspirin did not increase the incidence of overall cancer or CRC in ASPREE [6]. Taken together, an increase in cancer death after just 4.7 years of exposure without anatomic specificity or a corresponding increase in cancer incidence weakens the case that the association of aspirin with cancer mortality in ASPREE was causal.

Limitations of ASPREE as a cohort of older adults: In addition, the 2022 USPSTF did not consider the highly selected nature of the ASPREE cohort before weighing its results against prior RCTs that have shown a cancer benefit. Not only were ASPREE participants older (at least age 65-70 years), but the vast majority (85%) had never used aspirin. It should not be surprising that starting aspirin late in life might be too late to influence cancer initiation events that occur in mid-adulthood. Indeed, prior primary prevention trials suggest that the benefits of aspirin for CRC are confined to individuals who start aspirin at a younger age. For example, JPAD (Japanese Primary Prevention of Atherosclerosis with Aspirin for Diabetes) showed aspirin resulted in a hazard ratio (HR) for developing CRC of 0.41 (95% CI, 0.15-0.97) for those age less than 65 years [7]. A key study informing the 2016 USPSTF was long-term follow-up of the WHS (Women's Health Study), the largest primary prevention RCT of aspirin among women, which showed that CRC was reduced in those randomized to aspirin with a hazard ratio (HR) of 0.80 (95% CI, 0.67-0.97) after 10 years, with a posttrial HR of 0.58 (95% CI, 0.42-0.80). Importantly, even in WHS, there appeared to be potential modification of the effect by age: the aspirin group had a HR for CRC of 0.71 (95% CI, 0.52-0.98) among those age 45-54 years, with an attenuation of benefit for those who were older [8]. In our recent study of nearly 95,000 participants in the Nurses' Health Study and Health Professionals Follow-up Study, we prospectively examined aspirin use over

more than four decades and found that aspirin was associated with lower risk of CRC only if initiated before age 70 [9].

Need for USPSTF to consider prior data: Finally, the 2022 USPSTF did not weigh several well-conducted trials of aspirin showing a reduced risk of colorectal adenomas among individuals with a prior history of CRC or adenoma. These trials are compelling evidence of causality as adenomas are the precursors to the vast majority of CRC and an established surrogate endpoint for cancer. The 2022 USPSTF excluded these studies, reasoning that they applied only to "high-risk" populations. However, since adenomas are present in 20-30% of U.S. adults, many of whom are otherwise healthy, these trials should be considered as relevant as the "primary prevention" trials relied on by the 2022 USPSTF which included individuals selected for diabetes (ASCEND), moderate vascular risk (ARRIVE), or older age (ASPREE). The 2022 USPSTF also did not consider the recent results of CAPP2 (Colorectal Adenoma/carcinoma Prevention Program) which convincingly showed aspirin reduced CRC among participants Lynch syndrome after 10 years [10]. Although a "high-risk" population, these are the only trial data with CRC as a prespecified primary endpoint and provide compelling evidence of causality in the association of aspirin with CRC.

The need for precision prevention approaches: Thus, the evidence supporting that aspirin reduces risk of CRC does not appear to have substantially changed since the 2016 USPSTF recommendation. The 2022 USPSTF's reframing of the evidence on aspirin and CRC as "inadequate" is largely based on an unnuanced assessment of primary prevention studies. Notably, other expert bodies, including the American College of Gastroenterology and the American Gastroenterological Association [11, 12], considered the same available evidence as the USPSTF did, and they did not change their opinion about the value of aspirin for CRC prevention in 2021.

The shifting USPSTF recommendations underscore the pitfalls of interpreting imperfect data for cancer prevention in the interest of formulating broad recommendations based only on blunt categories of cardiovascular risk and age. It was perhaps unrealistic to expect that any cancer preventive agent, including aspirin, would make sense for all, or even most, individuals, particularly since resourcing and executing the requisite "one-size-fits-all" large-scale trials with long-term follow-up for cancer events are a major challenge. This underscores the importance of focusing future studies on "precision prevention" approaches that incorporate risk-stratifying individuals by either clinical or molecular factors to sharpen clinical recommendations to optimize risk-benefit. For example, starting aspirin at a younger age during an optimal biological window when cancers initiate yet bleeding risk is low may be a more impactful option than continuing the push to lower the age for screening eligibility to stem the rise in early onset CRC. In the meantime, the 2022 USPSTF guidelines unfortunately may serve to confuse patients and providers, potentially narrowing the option

for an individualized discussion of the risks and benefits. If so, this would be a disservice to advancing the field of cancer prevention for which there remains a high unmet need for low-cost, widely available agents with an established safety profile that can be used for the right person, at the right time, in the right way.

Aspirin and liver cancer: Additional opportunities: Metabolic dysfunction-associated steatotic liver disease (MASLD) represents the most common cause of chronic liver disease in Western countries, affecting over 30% of U.S. adults [13]. Up to one-third of patients with MASLD develop progressive steatohepatitis and fibrosis, which can lead to cirrhosis, hepatocellular carcinoma (HCC), and death [14, 15]. Despite significant investments in the development of therapies to treat steatotic liver disease (SLD), approved medications that effectively and safely reverse steatosis, Aspirin may represent a promising and low-cost strategy for treating MASLD and preventing progression to fibrosis, cirrhosis, and HCC. In preclinical studies, aspirin prevented intrahepatic platelet activation and Kupffer cell interactions, which are necessary for the development of steatohepatitis [16], and exhibited anti-inflammatory and anti-tumor effects, by inhibiting pro-inflammatory cyclooxygenase-2 (COX-2) and platelet-derived growth factor signaling [17], and by modulating bioactive lipids [18]. Consistent with those findings, observational studies associated aspirin use with a lower prevalence of hepatic steatosis [19], and with reduced rates of disease progression to advanced fibrosis [20]. We have also shown that aspirin is associated with lower risk of liver cancer in two separate cohorts [21, 22]. Thus, we recently completed a Phase II randomized clinical trial in adults with MASLD, to test the efficacy of low-dose aspirin compared to placebo, for reducing liver fat and markers of inflammation and fibrosis among adults aged 18-70 years with established MASLD without cirrhosis for 6 months. At the 2023 American Association for the Study of Liver Diseases, we reported that compared to placebo, aspirin significantly reduced absolute hepatic fat content, the primary outcome. Of four secondary outcomes, all were statistically significant, favoring aspirin. Nevertheless, given the current lack of approved treatments for MASLD, aspirin represents an attractive, accessible, and low-cost option, that now warrants further study. These data are currently submitted for publication. Taken together with the observational data that aspirin is associated with a lower risk of HCC, this trial provides causal data that aspirin can reduce hepatic fat, an established surrogate biomarker for fibrosis and eventual development of liver-related mortality, including liver cancer. These data support the need for further studies examining aspirin for HCC prevention, for which there is a high unmet need.

References

1. Drew DA, Chan AT. Aspirin in the prevention of colorectal neoplasia. *Annu Rev Med*.

2021;72(1):415-430. doi:10.1146/annurev-med-060319-120913
2. US Preventive Services Task Force, Davidson KW, Barry MJ, et al. Aspirin use to prevent cardiovascular disease: US Preventive Services Task Force recommendation statement. *JAMA*. 2022;327(16):1577. doi:10.1001/jama.2022.4983
3. Guirguis-Blake JM, Evans CV, Perdue LA, et al. Aspirin use to prevent cardiovascular disease and colorectal cancer: updated evidence report and systematic review for the US Preventive Services Task Force. *JAMA*. 2022;327(16):1585. doi:10.1001/jama.2022.3337
4. Chubak J, Kamineni A, Buist DS, et al. *Aspirin Use for the Prevention of Colorectal Cancer: An Updated Systematic Evidence Review for the US Preventive Services Task Force*. Agency for Healthcare Research and Quality (US); 2015. Accessed May 9, 2022. http://www.ncbi.nlm.nih.gov/books/NBK321661/
5. McNeil JJ, Nelson MR, Woods RL, et al. Effect of aspirin on all-cause mortality in the healthy elderly. *N Engl J Med*. 2018;379(16):1519-1528. doi:10.1056/NEJMoa1803955
6. McNeil JJ, Gibbs P, Orchard SG, et al. Effect of aspirin on cancer incidence and mortality in older adults. *JNCI: Journal of the National Cancer Institute*. 2021;113(3):258-265. doi:10.1093/jnci/djaa114
7. Ogawa H. Nakayama M, Morimoto, T, et al. Low-dose aspirin for primary prevention of atherosclerotic events in patients with type 2 diabetes: a randomized controlled trial. *JAMA*. 2008;300(18):2134. doi:10.1001/jama.2008.623
8. Cook NR, Lee IM, Zhang SM, et al. Alternate-day, low-dose aspirin and cancer risk: long-term observational follow-up of a randomized trial. *Ann Intern Med*. 2013;159(2):77. doi:10.7326/0003-4819-159-2-201307160-00002
9. Guo CG, Ma W, Drew DA, et al. Aspirin use and risk of colorectal cancer among older adults. *JAMA Oncol*. 2021;7(3):428. doi:10.1001/jamaoncol.2020.7338
10. Burn J, Sheth H, Elliott F, et al. Cancer prevention with aspirin in hereditary colorectal cancer (Lynch syndrome), 10-year follow-up and registry-based 20-year data in the CAPP2 study: a double-blind, randomised, placebo-controlled trial. *The Lancet*. 2020;395(10240):1855-1863. doi:10.1016/S0140-6736(20)30366-4
11. Shaukat A, Kahi CJ, Burke CA, Rabeneck L, et al. ACG clinical guidelines: colorectal cancer screening 2021. *Am J Gastroenterol*. 2021;116(3):458-479. doi:10.14309/ajg.0000000000001122
12. Liang PS, Shaukat A, Crockett SD. AGA clinical practice update on chemoprevention for colorectal neoplasia: expert review. *Clinical Gastroenterology and Hepatology*. 2021;19(7):1327-1336. doi:10.1016/j.cgh.2021.02.014
13. Riazi K, Azhari H, Charette JH, et al. The prevalence and incidence of NAFLD worldwide: a systematic review and meta-analysis. *Lancet Gastroenterol Hepatol* 2022;7(9):851-61. doi: 10.1016/S2468-1253(22)00165-0 [published Online First: 2022/07/08]

14. Vernon G, Baranova A, Younossi ZM. Systematic review: the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. *Aliment Pharmacol Ther* 2011;34(3):274-85. doi: 10.1111/j.1365-2036.2011.04724.x [published Online First: 2011/06/01]
15. Simon TG, Roelstraete B, Khalili H, et al. Mortality in biopsy-confirmed nonalcoholic fatty liver disease: results from a nationwide cohort. *Gut* 2020 doi: 10.1136/gutjnl-2020-322786 [published Online First: 2020/10/11]
16. Malehmir M, Pfister D, Gallage S, et al. Platelet GPIbalpha is a mediator and potential interventional target for NASH and subsequent liver cancer. *Nat Med* 2019;25(4):641-55. doi: 10.1038/s41591-019-0379-5 [published Online First: 2019/04/03]
17. Paik YH, Kim JK, Lee JI, et al. Celecoxib induces hepatic stellate cell apoptosis through inhibition of Akt activation and suppresses hepatic fibrosis in rats. *Gut* 2009;58(11):1517-27. doi: 10.1136/gut.2008.157420 [published Online First: 2009/02/10]
18. Gilligan MM, Gartung A, Sulciner ML, et al. Aspirin-triggered proresolving mediators stimulate resolution in cancer. *Proc Natl Acad Sci U S A* 2019;116(13):6292-97. doi: 10.1073/pnas.1804000116 [published Online First: 2019/03/14]
19. Shen H, Shahzad G, Jawairia M, et al. Association between aspirin use and the prevalence of nonalcoholic fatty liver disease: a cross-sectional study from the Third National Health and Nutrition Examination Survey. *Aliment Pharmacol Ther* 2014;40(9):1066-73. doi: 10.1111/apt.12944 [published Online First: 2014/09/02]
20. Simon TG, Henson J, Osganian S, et al. Daily Aspirin Use Associated With Reduced Risk For Fibrosis Progression In Patients With Nonalcoholic Fatty Liver Disease. *Clin Gastroenterol Hepatol* 2019;17(13):2776-84 e4. doi: 10.1016/j.cgh.2019.04.061 [published Online First: 2019/05/12]
21. Simon TG, Duberg AS, Aleman S, et al. Association of Aspirin with Hepatocellular Carcinoma and Liver-Related Mortality. *N Engl J Med* 2020;382(11):1018-28. doi: 10.1056/NEJMoa1912035 [published Online First: 2020/03/12]
22. Simon TG, Ma Y, Ludvigsson JF, Chong DQ, Giovannucci EL, Fuchs CS, Meyerhardt JA, Corey KE, Chung RT, Zhang X, Chan AT.* Association between aspirin use and risk of hepatocellular carcinoma. JAMA Oncol. 2018 Oct 4. PMCID: PMC6440745.

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ENVIRONMENTAL IMPACT ON THE GUT MICROBIOME: MULTI-OMICS TO MECHANISMS OF DIETARY SPINACH

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Complex interrelationships govern the dynamic interactions between gut microbes, the host, and exogenous drivers of disease outcome. A multi-omics approach to cancer interception by dietary spinach (SPI) was pursued [1, 2] via integrated metagenomic, transcriptomic and metabolomic studies in the polyposis in rat colon (Pirc) model [3]. SPI fed for 26 weeks (10% wt/wt, freeze-dried in the diet) exhibited significant antitumor efficacy in the colon and duodenum. Notably, in the Apc-mutant genetic background, β-catenin remained over-expressed in adenomatous polyps. In both wild type and Apc-mutant rats, increased gut microbiome diversity after 26-week dietary SPI consumption coincided with reversal of taxonomic composition. Reshaping of the gut microbiome was not evident after 3-day SPI intake, requiring >1-2 weeks to approximate the increased α-diversity observed at 26 weeks. By design, the fiber content was kept identical among the AIN-93 custom diets, and alternative mechanisms of action were sought.

Untargeted metabolomics corroborated metagenome prediction following chronic SPI intake: Metagenomic data implicated linoleate and butanoate metabolism, tricarboxylic acid cycle, and pathways in cancer, which was supported by transcriptomic and metabolomic analyses. Thus, tumor suppression by SPI involved marked reshaping of the gut microbiome and changes in host RNA-miRNA networks. When colon polyps were compared with matched normal-looking tissues via metabolomics, anticancer outcomes were linked to SPI-derived linoleate bioactives with known proapoptotic/anti-inflammatory mechanisms [4], as well as *N*-aceto-2-hydroxybutanoate, consistent with altered butanoate metabolism arising from increased α-diversity of the gut microbiome.

Mechanistic studies after chronic SPI consumption supported HDAC inhibition and

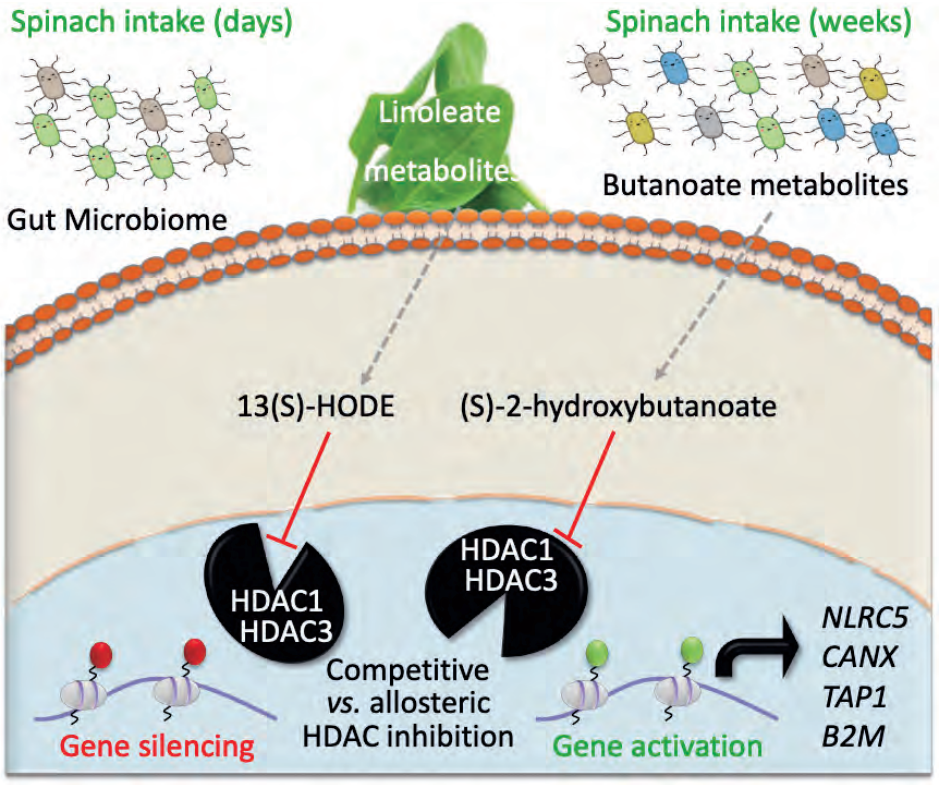

Fig. 1 HDAC inhibition and altered interferon-γ signaling by dietary spinach.

altered interferon-γ signaling: Follow-up mechanistic studies were undertaken in human and murine colon cancer cells. In addition to corroborating the HDAC inhibitory activity of single and combined metabolites, 13(S)-hydroxyoctadecadienoic acid (13(S)-HODE) and (S)-2-hydroxybutanoate elevated β2m protein levels, increased cell surface β2m expression, and induced IL-2 secretion from T-cell hybridomas co-cultured with colon cancer cells, consistent with enhanced MHC-I cell surface presentation. A working model proposes downregulation of MHC class I-dependent antigen presentation via epigenetic silencing mechanisms that were reversed by SPI-derived linoleate and gut microbiome-associated hydroxylated butanoate metabolites (Fig. 1). Immunoepigenetic regulation at the adenoma stage warrants further investigation with a view to delaying colectomy and drug intervention in at-risk patients [5, 6].

References

1. Chen YS, Menon R, Jayaraman A, Lee K, Huang Y, Dashwood WM, Zhang K, Sun D, Dashwood RH. Dietary spinach reshapes the gut microbiome in an Apc-mutant genetic

background: mechanistic insights from integrated multi-omics. *Gut Microbes*, 2021, 13, 1972756.

2. Chen, YS, Li J, Neja S, Kapoor S, Tovar Perez JE, Tripathi C, Menon R, Jayaraman A, Lee K, Dashwood WM, Wang S, Zhang K, Kobayashi K, Rajendran P, Dashwood R. Metabolomics of acute vs. chronic spinach intake in an Apc-mutant genetic background: linoleate and butanoate metabolites targeting HDAC activity and IFN-γ signaling. *Cells*, 2022, 11, 573.
3. Amos-Landgraf JM, Kwong LN, Kendziorski CM, Reichelderfer M, Torrealba J, Weichert J, Haag JD, Chen K-S, Waller JL, Gould MN, Dove WF. A target-selected Apc-mutant rat kindred enhances the modeling of familial human colon cancer. *Proc. Natl. Acad. Sci. U.S.A.*, 2007, 104, 4036-4041.
4. Liu F, Zuo X, Liu Y, Deguchi Y, Moussalli MJ, Chen W, Yang P, Wei B, Tan L, Lorenzi PL, Gao S, Jaoude JC, Mehdizadeh A, Valentin LA, Wei D, Shureiqi I. Suppression of membranous LRP5 recycling, Wnt/β-catenin signaling, and colon carcinogenesis by 15-LOX-1 peroxidation of linoleic acid in PI3P. *Cell Rep*, 2020, 32, 108049.
5. Ulusan AM, Rajendran P, Dashwood WM, Yavuz OF, Kapoor S, Gustafson TA, Savage MI, Brown PH, Sei S, Mohammed A, Vilar E, Dashwood RH. Optimization of erlotinib plus sulindac dosing regimens for intestinal cancer prevention in an Apc-mutant model of familial adenomatous polyposis (FAP). *Cancer Prev Res (Phila)*, 2021, 14, 325-336.
6. Samadder NJ, Foster N, McMurray RP, Burke CA, Stoffel E, Kanth P, Das R, Cruz-Correa M, Vilar E, Mankaney G, Buttar N, Thirumurthi S, Turgeon DK, Sossenheimer M, Westover M, Richmond E, Umar A, Della'Zanna G, Rodriguez LM, Szabo E, Zahrieh D, Limburg PJ. Phase II trial of weekly erlotinib dosing to reduce duodenal polyp burden associated with familial adenomatous polyposis. *Gut*, 2023, 72, 256-263.

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ADDUCTOMIC STRATEGIES FOR EMERGING RISK FACTORS IN LIVER CANCER

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Liver cancer and its impact on different populations and communities. An epidemiologic transition underpinning etiology of liver cancer is underway impacting concepts of cancer prevention and control interventions for this nearly always fatal disease. The proportional contributions of traditional etiological factors in HCC - hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol, aflatoxin and smoking - are changing with the emergence of metabolic syndrome, obesity, non- alcoholic fatty liver disease (NAFLD), air pollution, and diabetes. Overall, liver cancer, about 85% is a hepatocellular carcinoma (HCC) diagnosis, accounts for up to 8.2% of all reported cancer deaths and is among the top three causes of cancer mortality worldwide (1).

The global distribution of liver cancer incidence varies enormously and unfortunately the burden of this nearly always fatal disease is frequently much greater in low- and middle income countries (2). HCC has risen rapidly over the past 20 years in the US and Central America, and this poses an important public health and clinical problem for its diagnosis and management (3-6). In our continuing work in Central America where liver cancer is high and HBV and HCV infections are low, aflatoxin continues to be a major risk factor for HCC (7). In the most recent report on Cancer Health Disparities in the US the American Association for Cancer Research outlined that Hispanics have twice the rate of liver cancer mortality compared to other ethnic groups (8).

Sex differences in liver cancer incidence have been well documented and worldwide the number of cases is almost 600,000 among men and 250,000 among women in the latest data reported in 2020 (1). Sex differences are also evident in experimental animal data for liver carcinogens, exemplified by aflatoxin, where male rats have been found to have an earlier

onset and higher incidence of cancer compared to female animals (9, 10). Thus, the consistency of the experimental animal and human data points to the important role that environmental exposures play in sex differences in HCC risk. In many high-risk populations for HCC in Asia and Africa there has been a 2 to 8-fold greater prevalence of HCC in men compared to women (gco.iarc.fr). Remarkably in Central American countries, where liver cancer rates are the highest in the Western Hemisphere, the incidence in men and women is almost equivalent (gco.iarc.fr) (11). These findings compel more detailed investigations to discern environmental, dietary, and genetic risk factors that contribute to the rise of HCC and its remarkable impact for women.

An emerging risk for liver cancer risk comes from several epidemiologic studies reporting that ambient air pollution exposure, particularly particulate matter $PM_{2.5}$, increases the risk of liver cancer (12-15). A US-based ecological study found positive associations with ambient $PM_{2.5}$ levels (adjusted IRR per 10 $\mu g/m^3$: 1.26, 95% CI 1.08-1.47) (15). Similar results are reported from a prospective cohort study in Taiwan, with increased risk for HCC (adjusted HR per IQR [0.73 $\mu g/m^3$]: 1.22, 95% CI 1.02-1.47) (12). As part of the European Study of Cohorts for Air Pollution Effects (ESCAPE) project, there were suggestive associations for air pollution exposure and liver cancer risk for NO_2 and $PM_{2.5}$ (13). Finally, the Effects of Low-Level Air Pollution: A Study in Europe (ELAPSE) project showed increased risks with higher levels of exposure to NO_2 (adjusted HR per 10 $\mu g/m^3$: 1.17, 95% CI 1.02-1.35), and $PM_{2.5}$ (adjusted HR per 5 $\mu g/m^3$: 1.12, 95% CI 0.92-1.36) (14). In Central America both indoor and outdoor air pollution events have been well documented (16, 17). Collectively, these reports motivate the proposed research utilizing our albumin adductomics technology to assess air pollution exposures (18, 19).

Our team has developed an albumin adductomics approach employing nanoflow liquid chromatography-high resolution mass spectrometry and parallel reaction monitoring capable of simultaneously monitoring dozens of Cys^{34} and other amino acid adducts, in the low femtomolar range and using less than 2.5 μL of plasma (18, 19). We characterized the magnitude and impact of ambient outdoor air pollution exposures with three repeated measurements over 84 days in non- smoking women. In concordance with seasonally rising ambient concentrations of NO_2, SO_2, and PM_{10} measured at stationary monitors, we observed elevations in concentrations of Cys^{34} adducts of benzoquinone ($p<0.05$), benzene diol epoxide (BDE; $p<0.05$), crotonaldehyde ($p<0.01$), and oxidation ($p<0.001$). Regression analysis revealed significant elevations in oxidation and BDE adduct concentrations of 300% to nearly 700% per doubling of ambient airborne pollutant levels ($p<0.05$). Notably, the ratio of irreversibly oxidized to reduced Cys^{34} rose more than 3-fold during the 84-day period, revealing a dramatic perturbation of serum redox balance; potentially serving as a portent of increased pollution-related mortality risk. These oxidation biomarkers are now validated for

these proposed investigations.

Aflatoxin B_1 remains a major risk factor for liver cancer. Aflatoxin B_1 (AFB_1) is not directly carcinogenic but must first be transformed *in vivo* to its ultimate carcinogenic derivative. The ultimate carcinogenic metabolite of AFB_1, AFB_1-*exo*-8,9-epoxide, is produced in the liver through oxidation by cytochrome P450s (20). These activating enzymes are not rate limiting and there is a linear increase in adduct formation across dietary exposures (21). The AFB_1-*exo*-8,9-epoxide intercalates with DNA, facilitating electrophilic attack on guanine residues at the N^7 position. While the cytochrome P450s produces both an AFB_1 -8,9-*exo*- and AFB_1 -8,9-*endo*-epoxide, the *exo* epoxide is ~10^3 times as mutagenic as the *endo*, which exhibits negligible reactivity towards double-stranded DNA (22). The AFB_1-*exo*-8,9-epoxide is ultimately responsible for the production of the codon 249 mutation in p53 (10). This pathway also is critical in the production of a metabolically activated aflatoxin adduct with lysine in albumin and is a biomarker that tracks with DNA damage (23-26).

Many case-control studies have examined AFB_1 exposure and HCC, recently summarized (7). Data obtained from cohort studies have the greatest power to determine a true relationship between an exposure and disease. Using the aflatoxin biomarkers developed in our laboratory we demonstrated the important role of this AFB_1 exposure in the etiology of HCC. This work, comprising more than 18,000 men in Shanghai, revealed a statistically significant increase in the adjusted relative risk of 3.4 for HCC when AFB_1 biomarkers were detected. For subjects with positive HBsAg detection (a marker of infection with HBV), the relative risk was 7, but for individuals with both urinary aflatoxins and positive HBsAg status, the relative risk was 59 (27, 28). These results contributed to IARC classifying aflatoxin as a Group 1 human carcinogen (10). Further, these findings promote the hypothesis that other aflatoxin risk factor interactions could exist that contribute to HCC.

Mechanistic studies have demonstrated that AFB_1 mediated DNA damage causes induction of a guanine to thymine (G→T) transversion mutation in both human samples and experimental models. This lesion produces a characteristic mutational signature, particularly in p53 (29,30). Two initial reports found the presence of a G→T transversion hotspot at the third position of codon 249 (5′- CGG **AG<u>G</u>** CCC-3′) of the human *TP53* gene (31, 32), which results in the non-synonymous substitution of arginine with serine (R249S) within the p53 protein. These findings have been replicated in many settings of high AFB_1 exposure (33, 34) and data from our team found these same p53 mutations in HCCs from Guatemala and in a collaborative study in Mexico (35, 36).

Aflatoxin-albumin biomarkers document a reduction of liver cancer incidence by more than 50% in the absence of HBV vaccination. Validated aflatoxin specific biomarkers not only define exposure but also can define risk and in turn this work has led to the application of these biomarkers in preventive intervention trials (37, 38). The aflatoxin-albumin

biomarkers permitted us to characterize a concordance of a nearly 4000% reduction in exposure and a 50% reduction in liver cancer incidence over a 30-year time frame in Qidong China (39). Indeed, the reduction in aflatoxin exposure documented in this work preceded HBV vaccination in the population. Thus, the discovery of the powerful biologic role of aflatoxin in liver cancer development sets the stage for multiple public health-based prevention strategies. Given the data that we have ascertained in Guatemala to date implementing both primary and secondary (40) strategies in this high aflatoxin exposure, but low HBV and HCV prevalent region, could mitigate liver cancer.

Cell-free DNA analysis for early detection of liver cancer. Over the past ten years the translation of the underlying mechanistic knowledge of cancer has led to an emergence of liquid biopsy strategies for early detection and clinical care follow up (41). Recently Velculescu and Foda has pioneered the detection and measurement of genome wide landscape of cfDNA fragmentation (fragmentomes) and DELFI (DNA evaluation of fragments for early interception) (42, 43) was demonstrated to be strongly predictive for liver cancer in samples obtained from Europe, the US and Hong Kong (44). This machine learning model incorporated multi-feature fragmentome data to evaluate 724 individuals; the sensitivity for detecting liver cancer was 88% in an average risk population at 98% specificity, and 85% among high-risk individuals at 80% specificity (44). An independent study reported in 2022 in China found similar results using a technically similar fragmentomics strategy predicting liver cancer risk (45). In this recent study the ultimate model showed a 97% sensitivity and 96% specificity (45). Collectively the proposed work here provides an opportunity for extension and validation of these biomarkers to a different etiological risk group for early detection.

In summary, despite the recent successes in the prevention and treatment of HBV and HCV through vaccination and chemotherapy, the incidence of liver cancer continues to rise across many different populations. To affect future success in liver cancer prevention and control the identification of new underlying and modifiable risk factors are required. Well annotated epidemiologic studies and concomitant biorepositories will provide the foundational resource for this research.

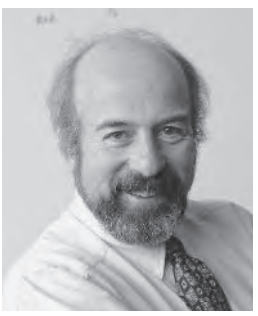
References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin **2021**;71:209-49
2. Wild CP, Miller JD, Groopman JD. Mycotoxin Control in Low- and Middle-Income Countries. **2015**

3. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, *et al*. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer **2015**;136:E359-86. doi: 10.1002/ijc.29210. Epub 2014 Oct 9.
4. El-Serag HB, Kanwal F. - Epidemiology of hepatocellular carcinoma in the United States: where are we? - Hepatology 2014 Nov;60(5):1767-75 doi: 101002/hep27222 Epub 2014 Aug 25 **2014**:- 1767-75
5. Petrick JL, Kelly SP, Altekruse SF, McGlynn KA, Rosenberg PS. Future of Hepatocellular Carcinoma Incidence in the United States Forecast Through 2030. J Clin Oncol **2016**
6. McGlynn KA, Petrick JL, London WT. Global epidemiology of hepatocellular carcinoma: an emphasis on demographic and regional variability. Clin Liver Dis **2015**;19:223-38. doi: 10.1016/j.cld.2015.01.001. Epub Feb 26.
7. Groopman JD, Smith JW, Rivera-Andrade A, Alvarez CS, Kroker-Lobos MF, Egner PA, *et al*. Aflatoxin and the Etiology of Liver Cancer and Its Implications for Guatemala. World Mycotoxin J **2021**;14:305-17
8. Cancer Disparities Progress Report. American Association for Cancer Research; **2022**. 200p.
9. Wogan GN, Newberne PM. Dose-response characteristics of aflatoxin B1 carcinogenesis in the rat. Cancer Res **1967**;27:2370-6
10. Kensler TW, Roebuck BD, Wogan GN, Groopman JD. Aflatoxin: a 50-year odyssey of mechanistic and translational toxicology. Toxicol Sci **2011**;120 Suppl 1:S28-48
11. Pineros M, Frech S, Frazier L, Laversanne M, Barnoya J, Garrido C, *et al*. Advancing Reliable Data for Cancer Control in the Central America Four Region. J Glob Oncol **2018**;4:1-11
12. Pan W-C, Wu C-D, Chen M-J, Huang Y-T, Chen C-J, Su H-J, Yang H-I. Fine particle pollution, alanine transaminase, and liver cancer: A Taiwanese prospective cohort study (REVEAL-HBV). Journal of the National Cancer Institute **2016**;108:djv341
13. Pedersen M, Andersen ZJ, Stafoggia M, Weinmayr G, Galassi C, Sørensen M, *et al*. Ambient air pollution and primary liver cancer incidence in four European cohorts within the ESCAPE project. Environmental Research **2017**;154:226-33
14. So R, Chen J, Mehta AJ, Liu S, Strak M, Wolf K, *et al*. Long-term exposure to air pollution and liver cancer incidence in six European cohorts. Int J Cancer **2021**
15. VoPham T, Bertrand KA, Tamimi RM, Laden F, Hart JE. Ambient PM2.5 air pollution exposure and hepatocellular carcinoma incidence in the United States. Cancer Causes Control **2018**;29:563-72
16. Smith KR, McCracken JP, Weber MW, Hubbard A, Jenny A, Thompson LM, *et al*. Effect of reduction in household air pollution on childhood pneumonia in Guatemala (RESPIRE): a randomised controlled trial. The Lancet **2011**;378:1717-26
17. Landrigan PJ, Fuller R, Acosta NJR, Adeyi O, Arnold R, Basu NN, *et al*. The Lancet

Commission on pollution and health. Lancet **2018**;391:462-512
18. Smith JW, O'Meally RN, Ng DK, Chen JG, Kensler TW, Cole RN, Groopman JD. Biomonitoring of Ambient Outdoor Air Pollutant Exposure in Humans Using Targeted Serum Albumin Adductomics. Chem Res Toxicol **2021**;34:1183-96
19. Smith JW, O'Meally RN, Burke SM, Ng DK, Chen JG, Kensler TW, *et al*. Global Discovery and Temporal Changes of Human Albumin Modifications by Pan-Protein Adductomics: Initial Application to Air Pollution Exposure. J Am Soc Mass Spectrom **2023**;34:595-607
20. Kamala A, Kimanya M, Haesaert G, Tiisekwa B, Madege R, Degraeve S, *et al*. Local post-harvest practices associated with aflatoxin and fumonisin contamination of maize in three agro ecological zones of Tanzania. Food additives & contaminants Part A, Chemistry, analysis, control, exposure & risk assessment **2016**;33:551-9
21. Wild CP, Jiang YZ, Allen SJ, Jansen LA, Hall AJ, Montesano R. Aflatoxin-albumin adducts in human sera from different regions of the world. Carcinogenesis **1990**;11:2271-4
22. Smela ME, Currier SS, Bailey EA, Essigmann JM. The chemistry and biology of aflatoxin B(1): from mutational spectrometry to carcinogenesis. Carcinogenesis **2001**;22:535-45
23. Wild CP, Garner RC, Montesano R, Tursi F. Aflatoxin B1 binding to plasma albumin and liver DNA upon chronic administration to rats. Carcinogenesis **1986**;7:853-8
24. Wild CP, Hudson GJ, Sabbioni G, Chapot B, Hall AJ, Wogan GN, *et al*. Dietary intake of aflatoxins and the level of albumin-bound aflatoxin in peripheral blood in The Gambia, West Africa. Cancer Epidemiol Biomarkers Prev **1992**;1:229-34
25. Groopman JD, Hall AJ, Whittle H, Hudson GJ, Wogan GN, Montesano R, Wild CP. Molecular dosimetry of aflatoxin-N7-guanine in human urine obtained in The Gambia, West Africa. Cancer Epidemiol Biomarkers Prev **1992**;1:221-7
26. Groopman JD, Egner PA, Schulze KJ, Wu LS, Merrill R, Mehra S, *et al*. Aflatoxin exposure during the first 1000 days of life in rural South Asia assessed by aflatoxin B(1)-lysine albumin biomarkers. Food Chem Toxicol **2014**;74:184-9
27. Ross RK, Yuan JM, Yu MC, Wogan GN, Qian GS, Tu JT, *et al*. Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. Lancet **1992**;339:943-6
28. Qian GS, Ross RK, Yu MC, Yuan JM, Gao YT, Henderson BE, *et al*. A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in Shanghai, People's Republic of China. Cancer Epidemiol Biomarkers Prev **1994**;3:3-10
29. Chawanthayatham S, Valentine CC, 3rd, Fedeles BI, Fox EJ, Loeb LA, Levine SS, *et al*. Mutational spectra of aflatoxin B1 in vivo establish biomarkers of exposure for human hepatocellular carcinoma. Proc Natl Acad Sci U S A **2017**;114:E3101-E9
30. Fedeles BI, Essigmann JM. Impact of DNA lesion repair, replication and formation on the mutational spectra of environmental carcinogens: Aflatoxin B1 as a case study. DNA Repair (Amst) **2018**;71:12-22
31. Ozturk M. p53 mutation in hepatocellular carcinoma after aflatoxin exposure. Lancet

1991;338:1356-9
32. Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC. Mutational hotspot in the p53 gene in human hepatocellular carcinomas. Nature **1991**;350:427-8
33. Kucab JE, Zou X, Morganella S, Joel M, Nanda AS, Nagy E, *et al*. A Compendium of Mutational Signatures of Environmental Agents. Cell **2019**;177:821-36 e16
34. Zhang W, He H, Zang M, Wu Q, Zhao H, Lu LL, *et al*. Genetic Features of Aflatoxin-associated Hepatocellular Carcinomas. Gastroenterology **2017**
35. Alvarez CS, Ortiz J, Bendfeldt-Avila G, Xie Y, Wang M, Wu D, *et al*. Analysis of TP53 aflatoxin signature mutation in hepatocellular carcinomas from Guatemala: A cross-sectional study (2016- 2017). Health Sci Rep **2020**;3:e155
36. Lino-Silva LS, Lajous M, Brochier M, Santiago-Ruiz L, Melchor-Ruan J, Xie Y, *et al*. Aflatoxin levels and prevalence of TP53 aflatoxin-mutations in hepatocellular carcinomas in Mexico. Salud Publica Mex **2022**;64:35-40
37. Wang JS, Shen X, He X, Zhu YR, Zhang BC, Wang JB, *et al*. Protective alterations in phase 1 and 2 metabolism of aflatoxin B1 by oltipraz in residents of Qidong, People's Republic of China. J Natl Cancer Inst **1999**;91:347-54
38. Egner PA, Wang JB, Zhu YR, Zhang BC, Wu Y, Zhang QN, *et al*. Chlorophyllin intervention reduces aflatoxin-DNA adducts in individuals at high risk for liver cancer. Proc Natl Acad Sci U S A **2001**;98:14601-6
39. Chen JG, Egner PA, Ng D, Jacobson LP, Munoz A, Zhu Y-R, *et al*. Reduced Aflatoxin Exposure Presages Decline in Liver Cancer Mortality in an Endemic Region of China. Cancer Prevention Research **2013**;6:1038-45
40. Chemoprevention Working G, Sporn MB. Prevention on Cancer in the Next Millennium: Report of the Chemoprevention Wokring Group to the American Association for Cancer Research. Cancer Res **1999**;59:4743-58
41. Mattox AK, Bettegowda C, Zhou S, Papadopoulos N, Kinzler KW, Vogelstein B. Applications of liquid biopsies for cancer. Sci Transl Med **2019**;11
42. Mathios D, Johansen JS, Cristiano S, Medina JE, Phallen J, Larsen KR, *et al*. Detection and characterization of lung cancer using cell-free DNA fragmentomes. Nat Commun **2021**;12:5060
43. Cristiano S, Leal A, Phallen J, Fiksel J, Adleff V, Bruhm DC, *et al*. Genome-wide cell-free DNA fragmentation in patients with cancer. Nature **2019**;570:385-9
44. Foda ZH, Annapragada AV, Boyapati K, Bruhm DC, Vulpescu NA, Medina JE, *et al*. Detecting liver cancer using cell-free DNA fragmentomes. Cancer Discov **2023**;13:616-31
45. Zhang X, Wang Z, Tang W, Wang X, Liu R, Bao H, *et al*. Ultrasensitive and affordable assay for early detection of primary liver cancer using plasma cell-free DNA fragmentomics. Hepatology **2022**;76:317-29



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INHIBITION OF COLORECTAL CARCINOGENESIS IN FAMILIAL ADENOMATOUS POLYPOSIS

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To date, many candidate chemopreventive agents against colorectal cancer (CRC) have been presented from researchers in the field of basic science and epidemiological science. Although CRC is the most common cancer in Japan, no preventive drugs are in clinical use yet. The reason for this can be partly explained by the system of public health insurance for the whole nation, which does not allow the use of drugs for a presymptomatic status, i.e. the status before diagnosis. From the view of scientific research, the reason for this can be partly explained by the lack of infrastructure to perform cancer prevention trials in Japan.

It was reported, by Dr. Thun in 1991, that aspirin reduced the risk of either colorectal polyps or cancer . Afterwards, many studies using NSAIDs were performed, which suggested their usefulness as cancer chemopreventive agents. Aspirin is an inhibitor of cyclooxygenase that catalyzes the conversion of arachidonic acid to prostaglandins. In addition, Aspirin has been reported to possess many other functions, such as inhibition of NF-kappa B and beta-catenin transcriptional activities.

Now, CRC chemoprevention trials have been performed in Japan. We investigated the effects of low-dose (100 mg/day), enteric coated aspirin tablets administered for 2 years in a double-blinded, randomized, placebo-controlled clinical trial for patients with a single/multiple colorectal adenoma (1). As a result, we obtained the result that low-dose enteric-coated aspirin tablets reduced the recurrence of colorectal tumor development in an Asian population (after adjustment for potential confounders, we obtained a marginal and significant OR of 0.60). Afterwards, a CRC chemoprevention trial using metformin, an anti-diabetes drug, was performed at other facilities, and showed reduction of the recurrence of colorectal tumor development (2).

We established mode of action for the proof of concept (POC) of aspirin that it inhibits intestinal polyp development, before moving to the human trial. The areas being considered in this trial were, (i) Anti-inflammation, (ii) Anti-oxidative stress, and (iii) Improvement of abnormal beta-catenin signaling.

We believe that the use of aspirin as a cancer chemopreventive agent has advantages because it has a long history of clinical use and its adverse effects are well known. An overseas clinical trial investigated the effects of aspirin in familial adenomatous polyposis (FAP), using high-dose aspirin (CAPP1). This trial was conducted with a 2 × 2 factorial design (a type of randomized controlled trial) using aspirin (600 mg/day), resistant starch, or a respective placebo in young FAP patients aged 10 to 21 years at low risk of cardiovascular disorder. In this study, resistant starch had no effect on the occurrence of colorectal polyps, but aspirin showed a tendency to reduce, though not significantly, the number of polyps in the area from the sigmoid colon to the rectum.

We first performed a Phase I study using low-dose enteric-coated aspirin tablets (100 mg/day) for 6 months, and also evaluated the occurrence of new intestinal polyps in Japanese patients after polyp removal (J-FAPP Study II) (3). In this trial, 17 patients were allocated to each of the aspirin and placebo groups. The results showed that the aspirin group had a higher number of participants showing a decreased number of colorectal polyps (primary endpoint) (risk ratio = 2.33 [95% CI: 0.72-7.55]), which only suggested that aspirin appeared to be effective.

In the next trial, we performed a chemoprevention trial using low-dose aspirin, a 2 x 2 factorial, randomised, double-blind, placebo-controlled, multicenter trial (11 centers) for FAP in Japan. FAP is characterized by the development of more than 100 polyps in the colorectum, and thus, is a high-risk status/condition for CRC. FAP and Lynch syndrome are well-known hereditary colorectal cancers for which causative genes have been identified. FAP is an autosomal dominant inherited condition, and the *APC* tumor suppressor gene has been identified as the causative gene. Hereditary colorectal cancer accounts for approximately 5% of all diagnosed cases of colorectal cancer.1) FAP accounts for less than 1% of all diagnosed cases of CRC. To avoid development of CRC, FAP patients undergo colonic resection at the age of around 20. From our results, administration of low-dose aspirin for 8 months safely inhibited the growth of colorectal polyps of more than 5.0 mm in FAP (4).

We also confirmed the beneficial effects of intensive downstaging polypectomy (IDP; an endoscopic method that resects all colorectal polyps more than 5.0 mm) in FAP patients at a young age (5). For this endoscopic treatment, bipolar snare polypectomy is used in general. Hot biopsy or argon-dye laser is not used. We removed 100 to 400 polyps during one polypectomy procedure (in about 90 minutes), and repeated this several times. We recruited 223 patients in this trial. The patients were followed up for 5 years. During the 5 years, the

average number of endoscopies was 6.6 times in the non-colectomy group (n=150) and 6.2 times in the colectomy group (n=47). The average number of removals was 524 in the non-colectomy group and 132 in the colectomy group. There was no bleeding requiring transfusion, and no development of advanced CRC during the 5 years. IDP showed its safety and effectiveness in preventing colonic carcinogenesis.

I believe that a combination treatment of IDP & aspirin will be a useful method for FAP patients who decline to undergo surgery due to personal or social reasons, and for reducing frequent diarrhea that decreases quality of life (QOL) after colectomy. IDP could be used in combination with colectomy. The problem might be the cost and manpower caused by the number of endoscopy specialists required. However, aspirin will postpone the interval of IDP treatment, and may solve these problems. In 2022, IDP was approved in the system of public health insurance for the whole nation. Use of low-dose aspirin for cancer prevention is not allowed yet. Use of low-dose aspirin has been approved only as an antithrombotic or anticoagulant agent. Therefore, we are now planning our final study aimed towards obtaining National Health Insurance coverage using low-dose aspirin (J-FAPP Study V). In this multicenter, single-arm interventional clinical study, we will administer low-dose enteric-coated aspirin (100 mg/day) for 2 years in patients with FAP and evaluate its effects in reducing the incidence of colorectal polyps of 5.0 mm or greater in diameter.

Our results will propose novel remedies, alternative to colorectal resection, that can be offered to FAP patients who decline to undergo surgery, and may provide future prospects for chemoprevention of CRC with respect to the future development of precision medicine.

References

1. Ishikawa H, Mutoh M*, Suzuki S, Tokudome S, Saida Y, Abe T, Okamura S, Tajika M, Joh T, Tanaka S, Kudo S, Matsuda T, Iimuro M, Yukawa T, Takayama T, Sato Y, Lee K, Kitamura S, Mizuno M, Sano Y, Gondo N, Sugimoto K, Kusunoki M, Goto C, Matsuura N, Sakai T, Wakabayashi K. The preventive effects of low-dose enteric-coated aspirin tablets on the development of colorectal tumours in Asian patients: a randomized trial. *Gut* 2014, 63, 1755-1759. *corresponding author
2. Higurashi T, Hosono K, Takahashi H, Komiya Y, Umezawa S, Sakai E, Uchiyama T, Taniguchi L, Hata Y, Uchiyama S, Hattori A, Nagase H, Kessoku T, Arimoto J, Matsuhashi N, Inayama Y, Yamanaka S, Taguri M, Nakajima A. Metformin for chemoprevention of metachronous colorectal adenoma or polyps in post-polypectomy patients without diabetes: a multicentre double-blind, placebo-controlled, randomised phase 3 trial. *Lancet Oncol* 2016, 17, 475-483.
3. Ishikawa H, Wakabayashi K, Suzuki S, Mutoh M, Hirata K, Nakamura T, Takeyama I,

Kawano A, Gondo N, Abe T, Tokudome S, Goto C, Matsuura N, Sakai T. Preventive effects of low-dose aspirin on colorectal adenoma growth in patients with familial adenomatous polyposis: Double-blind, randomized clinical trial. *Cancer Med* 2013, 2, 50-56.

4. Ishikawa H, Mutoh M*, Sato Y, Doyama H, Tajika M, Tanaka S, Horimatsu T, Takeuchi Y, Kashida H, Tashiro J, Ezoe Y, Nakajima T, Ikematsu, H Hori S, Suzuki S, Otani T, Takayama T, Ohda Y, Mure K, Wakabayashi K, Sakai T. Chemoprevention with low-dose aspirin, mesalazine, or both in patients with familial adenomatous polyposis without previous colectomy (J-FAPP Study IV): a multicentre, double-blind, randomised, two-by-two factorial design trial. *Lancet Gastroenterol Hepatol*. 2021, 6, 474-481. *corresponding author
5. Ishikawa H, Yamada M, Sato Y, Tanaka S, Chino A, Tajika M, Doyama H, Takayama T, Ohda Y, Horimatsu T, Sano Y, Tanakaya K, Ikematsu H, Saida Y, Ishida H, Takeuchi Y, Kashida H, Kiriyama S, Hori S, Lee K, Tashiro J, Kobayashi N, Nakajima T, Suzuki S, Mutoh M*, and J-FAPP Study III Group. Intensive endoscopic resection for downstaging of polyp burden in patients with familial adenomatous polyposis (J-FAPP Study III): A multicenter prospective interventional study. *Endoscopy* 2023, 55, 344-352. *corresponding author

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MICROBES AND COLON CANCER: WHERE ARE WE ?

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Introduction

Colon cancer is a leading cause of cancer morbidity and mortality of women and men across the globe (Siegel et al., 2023). How the vast microbial world of the colon impacts the initiation and/or progression of human colon cancer is an evolving field of investigation. Most of the focus to date—and the focus of this presentation—is on the putative contribution of the trillions of colon bacteria to colon carcinogenesis. From a broad perspective, key considerations are how single bacteria, presumably through specific virulence factors, or how bacterial communities, presumably through their interactions, may initiate and/or augment the progression of colon cancer (**Figure 1**). Most likely, both specific bacteria and community interactions govern colon carcinogenesis in combination with a broad array of host factors (e.g., obesity, smoking, alcohol intake, diabetes mellitus, prior antibiotic exposure among others), each known to also exert impact on the colon microbiome of individuals (Knippel et al., 2021). Thus, ultimately, integrating considerations of the host's colon microbiome into preventive strategies may prove to be a powerful way to improve colon cancer prevention. Herein, a brief presentation of both the power of individual bacterial

species and the colon bacterial community to influence colon carcinogenesis will be presented.

The Power of Individual Bacterial Species to Impact Colon Carcinogenesis

To date, we estimate that published data on 11 bacterial species suggest their association with colon cancer (**Figure 2**)(White & Sears, 2023). Among these species, *Bacteroides fragilis* toxin (BFT)-secreting *B. fragilis* strains, termed enterotoxigenic *B. fragilis* (ETBF), polyketide synthase-positive *Escherichia coli* strains (*pks+ E. coli*) and *Fusobacterium nucleatum* and its subspecies have garnered the most attention. Historically, detection of bloodstream infection with *Streptococcus gallolyticus* (formerly S. bovis, type I) was the first bacterium recognized to serve as a clinical signal worrisome for underlying colon cancer. In fact, it remains standard-of-care that detection of *S. gallolyticus* bloodstream infection requires a follow-up colonoscopy to rule out the co-occurrence of colon cancer (White & Sears, 2023). Herein, the data supporting the potential of ETBF and *pks+ E. coli* to enhance colon carcinogenesis will be described. The review by White and Sears (2023) provides a recent discussion of the spectrum of bacteria associated with colon carcinogenesis.

Through a series of elegant deductive experimental work conducted by Dr. Lyle Myers of Montana State University, ETBF was first identified as a cause of diarrhea in husbandry animals including lambs, calves and piglets among others; work that was subsequently replicated in lamb and rabbit animal models [reviewed in (Sears et al., 1995)]. Dr. Myers first identified that the culture supernatants of ETBF contained a heat-labile protein (BFT) that was capable of stimulating secretion in the ligated ileal loops of lambs (Sears et al., 1995). Subsequent work by the Sears laboratory and others identified an in vitro assay for detection of the BFT that assisted with identification of the *B. fragilis* gene encoding *bft* and purification of biologically active BFT protein [reviewed in (Sears et al., 2014)]. Through these results and others, BFT was defined as a pre-pro-zinc-dependent metalloprotease protein with, in particular, the pro-protein domain of BFT being critical to folding and delivery of biologically-active BFT to the external environment. This work led to the recognition that *B. fragilis* were composed of, at least, two molecular subtypes, ETBF and non-toxigenic *B. fragilis* (NTBF). Overall, NTBF is considered to contribute to colon health and acts as a symbiote whereas ETBF is recognized to be associated with inflammatory diarrheal disease, inflammatory bowel disease and human sporadic and hereditary colon cancer (Sears et al., 2014)(Boleij et al., 2015))(Dejea et al., 2018).

Key features of the pathogenesis of ETBF-associated colon tumorigenesis are shown in **Figure 3**. This model of the mechanisms by which ETBF, through BFT secretion, promotes colon tumor formation is based on extensive studies of ETBF pathogenicity using the multiple intestinal neoplasia (Min, $Apc^{+/-}$) murine model of colon tumorigenesis (White &

Sears, 2023) (Sears et al., 2014) (Chung et al., 2018). While mucosal adherence is undoubtedly critical to the pathogenicity of ETBF, in-frame deletion of *bft* is sufficient to eliminate the colon tumor-promoting activity of ETBF (Chung et al., 2018). Other mechanisms associated with ETBF colon tumorigenesis include cleavage of E-cadherin, induction of c-Myc expression leading to colon epithelial cell proliferation, induction of DNA damage and activation of Stat3 with a marked increased in IL-17 production by the colon mucosal immune cells (both Th17 and γδ-T cells) (Housseau et al., 2016). Feedback of IL-17 onto the colon epithelial cells results in NF-κB activation, chemokine secretion and distal colon infiltration by pro-tumor myeloid cells (e.g., myeloid-derived suppressor cells, MDSCs), also contributors to ETBF colon tumorigenesis (Chung et al., 2018). Collectively, in the Min mouse colon, these molecular sequelae rapidly lead to microadenoma formation that evolves to macroadenoma formation. More recently, the combined presence of colon cancer-associated oncogenes (*Apc*, *BRAF*) in a murine model has been identified to further modify ETBF pathogenesis (Destefano Shields et al., 2021), suggesting that there is the potential for dynamic interactions of colon microbial members, the oncogenes of colon cancer and its immune microenvironment (Dzierozynski et al., 2023). A key gap in knowledge is that the colon epithelial cell receptor for BFT remains to be identified.

An impressive series of publications have highlighted the potential contributions of *pks+ E. coli* to colon cancer development (Pleguezuelos-Manzano et al., 2020)(Wilson et al., 2019) (Dziubańska-Kusibab et al., 2020)(Xue et al., 2019)(Arthur et al., 2012.). The critical virulence determinant of *pks+ E. coli* is the synthesis of colibactin by the genes of the *pks* island. The structural elucidation of colibactin and the recognition of its striking, capacity to cross-link DNA leading to a specific mutational signature has generated much interest in the epidemiology of *pks+ E. coli* and their potential to initiate colon cancer. While it is hypothesized that the mutagenic capacity of, at least, some *pks+ E. coli* contribute to human colon cancer, longitudinal data defining the association of *pks+ E. coli* DNA mutagenesis to onset of neoplasia in the human colon are not yet available. However, *pks+ E. coli* can asymptomatically colonize children, raising the potential for putative long-term colonization with *pks+ E. coli*. This possibility has led to the hypothesis that, combined with host risk factors associated with human colon cancer, *pks+ E. coli* may contribute to the increase in early onset colorectal cancer, defined as a colon or rectal cancer diagnosis prior to age 50, when preventive colonoscopy is not routinely performed. Another clue to the importance of both ETBF and *pks+ E. coli* to colon cancer pathogenesis are observations in the hereditary colon cancer syndrome, familial adenomatous polyposis (FAP; autosomal dominant inheritance of mutation in one *APC* allele). In individuals with FAP, both ETBF and *pks+ E. coli* have been identified as prominent colonizers of mucus-invasive colon biofilms (see below) (Dejea et al., 2018).

The Power of Colon Bacterial Communities to Impact Colon Carcinogenesis

Visually colon mucosal biofilms display dense bacterial invasion of the normally sterile, Muc-2-dominated inner mucus layer (**Figure 1**). Specific criteria to identify a mucus-invasive biofilm are > 20 bacterial cells within 1 μm of the colon epithelial cell surface over a mucosal surface distance of, at least, 150-200 μm. These parameters define that ~10^9 or more bacterial cells are in proximity to the cell-of-origin for colon cancer, the colon epithelial cell. Available data have identified that biofilms are strongly associated with colon cancer (present on, at least, 50% of colon cancers both in the USA and Malaysia), are polymicrobial and are pro-carcinogenic in the Min mouse model (Dejea et al., 2014) (Drewes et al., 2017) (Tomkovich et al., 2019). In contrast, biofilms are found infrequently (~10-15%) in individuals undergoing preventive colonoscopy (Dejea et al., 2014). Whether individuals displaying mucus-invasive polymicrobial biofilms are at increased risk for development of colon neoplasia is, at present, unknown.

Recently, a consortium of bacteria isolated from the mucosa of a human colon cancer displaying a mucus-invasive, polymicrobial biofilm was studied (Drewes et al., 2022). This relatively small community of bacteria (N=30 strains) was shown to be pro-carcinogenic in both germ-free and specific pathogen-free Min mouse models. Additional studies, unexpectedly, identified that within this bacterial community *Clostridioides difficile*, through its secreted toxin B, was the key bacterial driver of the tumorigenesis observed in the Min murine model. Beyond toxin B secreted by *C. difficile*, the available data support that colon epithelial cell and immune mechanisms as well as biologic changes in *C. difficile*-associated murine colon biofilms contribute to the putative oncogenicity of *C. difficile*. Importantly, compared to Min mouse colonization with the biofilm isolates in the absence of *C. difficile*, inclusion of *C. difficile* in the colonizing consortium leads to biofilms that not only invade the inner mucus layer but invade the colon crypts, enhancing contact with colon stem cells. The proximity of toxin B-secreting *C. difficile* to colon stem cells is hypothesized to contribute to its tumorigenic ability in Min mice. Importantly, while *C. difficile* is the leading cause of nosocomial infection and a critical organism as defined by the Centers for Disease Control (*Antibiotic Resistance Threats in the United States*, 2019), its potential to contribute to colon carcinogenesis represents a new area for further investigation.

Summary and Conclusions

Given the complexity of the colon microbiota, it is not a surprise that multiple individual bacteria displaying or secreting oncogenic virulence factors and dense communities of bacteria, such as in colon mucosal biofilms, likely contribute to colon cancer pathogenesis. Dense bacterial communities are well-known to modify their function through inter-species signaling (such as by quorum sensing) (Oliveira et al., 2023); by extension, changes in the

function of nearby host cells as well as reciprocal interactions have been reported (Liou et al., 2022). While important strides have been made in unraveling the complex factors governing how the colon microbiota impact colon carcinogenesis, there remain substantive gaps in our understanding of the epidemiology, virulence, persistence and tumor-inducing potential of specific bacteria and communities in humans (White & Sears, 2023) (Knippel et al., 2021). Progress to utilization of colon microbiota biomarkers in the global quest to prevent colon cancer requires that we can discern the host context and bacterial strains or associated molecules that pose an oncogenic risk to an individual. These discoveries may offer a gateway to a new era in colon cancer prevention.

References

1. *Antibiotic resistance threats in the United States*, 2019. (2019). https://doi.org/10.15620/cdc:82532
2. Arthur, J. C., Perez-Chanona, E., Mühlbauer, M., et al. (2012) Intestinal Inflammation Targets Cancer-Inducing Activity of the Microbiota Science, 338, 120-123. http://*science*.sciencemag.org/
3. Boleij, A., Hechenbleikner, E. M., Goodwin, A. C., et al. (2015). The *Bacteroides fragilis* toxin gene is prevalent in the colon mucosa of colorectal cancer patients. *Clinical Infectious Diseases*, 60(2), 208–215. https://doi.org/10.1093/cid/ciu787
4. Chung, L., Thiele Orberg, E., Geis, A. L., et al. (2018). *Bacteroides fragilis* Toxin Coordinates a Pro-carcinogenic Inflammatory Cascade via Targeting of Colonic Epithelial Cells. *Cell Host and Microbe*, 23(2), 203-214.e5. https://doi.org/10.1016/j.chom.2018.01.007
5. Dejea, C. M., Fathi, P., Craig, J. M., et al (2018). Patients with familial adenomatous polyposis harbor colonic biofilms containing tumorigenic bacteria. *Science*, 359(6375), 592–597. https://doi.org/10.1126/science.aah3648
6. Dejea, C. M., Wick, E. C., Hechenbleikner, E. M., et al. (2014). Microbiota organization is a distinct feature of proximal colorectal cancers. *Proceedings of the National Academy of Sciences of the United States of America*, 111(51), 18321–18326. https://doi.org/10.1073/pnas.1406199111
7. Destefano Shields, C. E., White, J. R., Chung, L., et al. (2021). Bacterial-driven inflammation and mutant braf expression combine to promote murine colon tumorigenesis that is sensitive to immune checkpoint therapy. *Cancer Discovery*, 11(7), 1792–1807. https://doi.org/10.1158/2159-8290.CD-20-0770
8. Drewes, J. L., Chen, J., Markham, N. O., et al. (2022). Human Colon Cancer–Derived *Clostridioides difficile* Strains Drive Colonic Tumorigenesis in Mice. *Cancer Discovery*, 12(8), 1873–1885. https://doi.org/10.1158/2159-8290.CD-21-1273
9. Drewes, J. L., White, J. R., Dejea, et al. (2017). High-resolution bacterial 16S rRNA gene

profile meta-analysis and biofilm status reveal common colorectal cancer consortia. *Npj Biofilms and Microbiomes*, 3(1). https://doi.org/10.1038/s41522-017-0040-3

10. Dzierozynski, L., Queen, J., & Sears, C. L. (2023). Subtle, persistent shaping of the gut microbiome by host genes: A critical determinant of host biology. In *Cell Host and Microbe* (Vol. 31, Issue 10, pp. 1569–1573). Cell Press. https://doi.org/10.1016/j.chom.2023.09.007
11. Dziubańska-Kusibab, P. J., Berger, H., Battistini, F., et al. (2020). Colibactin DNA-damage signature indicates mutational impact in colorectal cancer. *Nature Medicine*, 26(7), 1063–1069. https://doi.org/10.1038/s41591-020-0908-2
12. Housseau, F., Wu, S., Wick, E. C., et al. (2016). Redundant Innate and Adaptive Sources of IL17 Production Drive Colon Tumorigenesis. *Cancer Research*, 76(8), 2115–2124. https://doi.org/10.1158/0008-5472.CAN-15-0749
13. Knippel, R. J., Drewes, J. L., & Sears, C. L. (2021). The cancer microbiome: Recent highlights and knowledge gaps. *Cancer Discovery*, 11(10), 2378–2395. https://doi.org/10.1158/2159-8290.CD-21-0324
14. Liou, M. J., Miller, B. M., Litvak, Y., et al. (2022). Host cells subdivide nutrient niches into discrete biogeographical microhabitats for gut microbes. *Cell Host and Microbe*, 30(6), 836-847.e6. https://doi.org/10.1016/j.chom.2022.04.012
15. Oliveira, R. A., Cabral, V., Torcato, I., & Xavier, K. B. (2023). Deciphering the quorum-sensing lexicon of the gut microbiota. In *Cell Host and Microbe* (Vol. 31, Issue 4, pp. 500–512). Cell Press. https://doi.org/10.1016/j.chom.2023.03.015
16. Pleguezuelos-Manzano, C., Puschhof, J., Rosendahl Huber, A., et al. (2020). Mutational signature in colorectal cancer caused by genotoxic *pks + E. coli*. *Nature*, 580(7802), 269–273. https://doi.org/10.1038/s41586-020-2080-8
17. Sears, C. L., Geis, A. L., & Housseau, F. (2014). *Bacteroides fragilis* subverts mucosal biology: From symbiont to colon carcinogenesis. In *Journal of Clinical Investigation* (Vol. 124, Issue 10, pp. 4166–4172). American Society for Clinical Investigation. https://doi.org/10.1172/JCI72334
18. Sears, C. L., Myers, L. L., Lazenby, A., & Van Tassell, R. L. (1995). Enterotoxigenic *Bacteroides fragilis*. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, 20 Suppl 2, S142-8. https://doi.org/10.1093/clinids/20.supplement_2.s142
19. Siegel, R. L., Miller, K. D., Wagle, N. S., & Jemal, A. (2023). Cancer statistics, 2023. CA: A *Cancer Journal for Clinicians*, 73(1), 17–48. https://doi.org/10.3322/caac.21763
20. Tomkovich, S., Dejea, C. M., Winglee, K., et al. (2019). Human colon mucosal biofilms from healthy or colon cancer hosts are carcinogenic. *Journal of Clinical Investigation*, 129(4), 1699–1712. https://doi.org/10.1172/JCI124196
21. White, M. T., & Sears, C. L. (2023). The microbial landscape of colorectal cancer. In *Nature Reviews Microbiology*. Nature Research. https://doi.org/10.1038/s41579-023-00973-4

22. Wilson, M. R., Jiang, Y., Villalta, P. W., et al. (2019). The human gut bacterial genotoxin colibactin alkylates DNA. *Science*, 363(6428). https://doi.org/10.1126/science.aar7785
23. Xue, M., Kim, C. S., Healy, A. R., et al. (2019). Structure elucidation of colibactin and its DNA cross-links. *Science*, 365(6457). https://doi.org/10.1126/science.aax2685

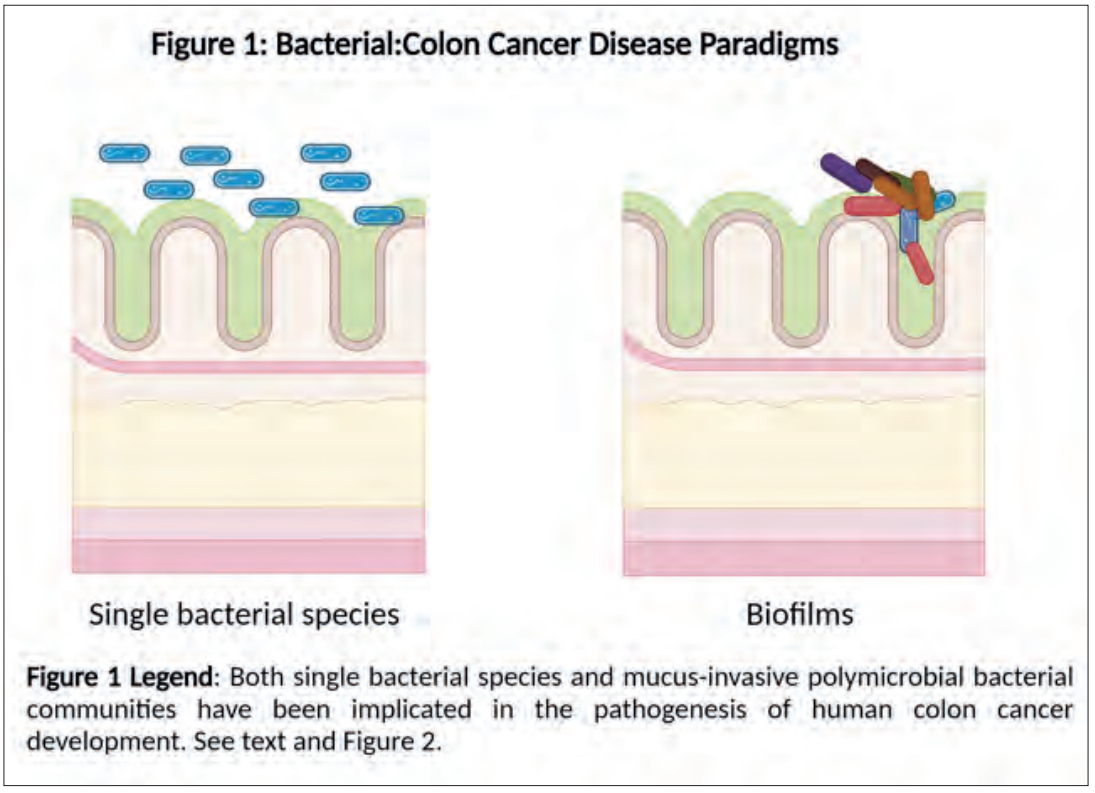

Figure 1 Legend: Both single bacterial species and mucus-invasive polymicrobial bacterial communities have been implicated in the pathogenesis of human colon cancer development. See text and Figure 2.

Figure 2
Assessing the Evidence: Bacteria Associated with Colon Cancer.

Bacteria associated with Colon Cancer	Epidemiologic Evidence	Murine Model with Colonization
pks+ Escherichia coli	*pks+ Escherichia coli*	*pks+ Escherichia coli*
bft+ Bacteroides fragilis	*bft+ Bacteroides fragilis*	*bft+ Bacteroides fragilis*
bft- Bacteroides fragilis	*bft- Bacteroides fragilis*	*bft- Bacteroides fragilis*
Fusobacterium nucleatum subspp	*Fusobacterium nucleatum subspp*	*Fusobacterium nucleatum subspp*
Streptococcus gallolyticus	*Streptococcus gallolyticus*	*Streptococcus gallolyticus*
Peptostreptococcus anaerobius	*Peptostreptococcus anaerobius*	*Peptostreptococcus anaerobius*
Porphyromonas gingivalis	*Porphyromonas gingivalis*	*Porphyromonas gingivalis*
Parvimonas micra	*Parvimonas micra*	*Parvimonas micra*
Salmonella enterica serovar Typhimurium	*Salmonella enterica serovar Typhimurium*	*Salmonella enterica serovar Typhimurium*
CDT+ Campylobacter jejuni	*CDT+ Campylobacter jejuni*	*CDT+ Campylobacter jejuni*
tcdB+ Clostridioides difficile	*tcdB+ Clostridioides difficile*	*tcdB+ Clostridioides difficile*

Figure 2 Legend. Currently, publications support that 11 bacterial species are associated with the pathogenesis of colon cancer (left box) (White and Sears, 2023). Epidemiologic evidence supports that the majority of these bacteria are associated with human colon cancer (middle box). Limited evidence supports the association of *Salmonella enterica serovar Typhimurium* with colon cancer (tan text, middle box). In contrast, for several of these bacteria, persistently colonized murine models after a single peroral bacterial gavage have been more difficult to establish for studies of the mechanisms of oncogenesis (right box). Bacteria in tan color represent microbes for which no murine is either yet described or murine models in which repeated gavage is utilized.

Figure 3: ETBF-associated colon tumorigenesis

$Apc^{+/-}$ murine model

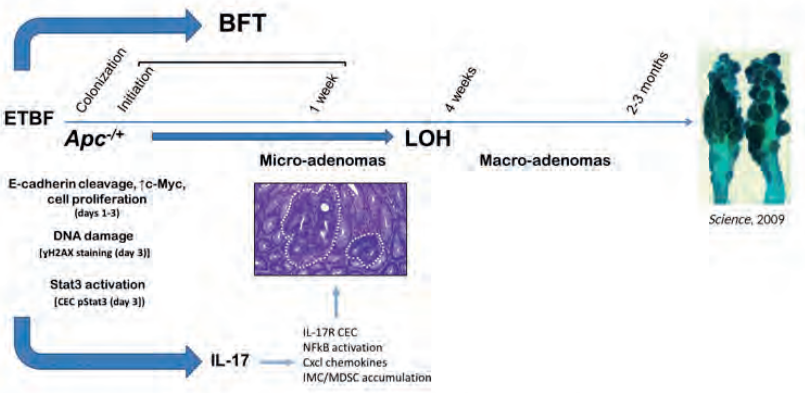

Figure Legend: Enterotoxigenic *Bacteroides fragilis* (ETBF) induces distal colon tumorigenesis in multiple intestinal neoplasia mice (Min, $Apc^{+/-}$) through release of the *B. fragilis* toxin (BFT) and stimulation of IL-17-dependent mucosal inflammation. BFT causes cleavage of the zonula adherens protein, E-cadherin, releasing its associated β-catenin triggering transcription of c-Myc and proliferation of colon epithelial cells (CEC) along with CEC DNA damage and CEC activation of Stat3. Increased mucosal IL-17 from CD4+ and γδ-T cells feeds back on CECs, resulting in CEC NFκβ activation and chemokine release that increase immature myeloid cells (IMC) and myeloid-derived suppressor cells (MDSC) in the distal colon. Distal colon micro-adenomas begin to form by 7 days after colonization of Min mice by ETBF that, over time, become macro-adenomas. $Apc^{+/-}$, heterozygous adenomatous polyposis coli gene. See text for additional details and references.

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COMPREHENSIVE GENOMIC PROFILING AND CLONAL EVOLUTION OF NEUROENDOCRINE CARCINOMAS OF THE GASTROINTESTINAL SYSTEM

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Neuroendocrine neoplasms (NENs) are rare disorders that can be diagnosed by exhibiting positive staining for neuroendocrine markers, such as synaptophysin and chromogranin A. NENs arise in a cross-organic manner. The diseases were classified into well-differentiated NENs (i.e., neuroendocrine tumors [NETs]), and poorly differentiated NENs (i.e., neuroendocrine carcinoma [NEC]), based on histopathology in the 2019 WHO classification.

Neuroendocrine carcinoma of the gastrointestinal system (GIS-NEC) is an uncommon yet highly aggressive tumor. Due to the frequent occurrence of distant metastases at diagnosis in GIS-NEC cases, surgical intervention is recommended in select patients. Consequently, there needs to be a more comprehensive analysis of genomic and epigenomic data obtained from frozen surgical samples. Due to the scarcity of frozen samples, we collected samples from Japan, the United States, and the Netherlands.The GIS-NEC cases were analyzed using whole-genome/exome sequencing, RNA-seq, DNA methylation assays, and the assay of transposase-accessible chromatin sequencing (ATAC) [1].

It is presumed that the majority of GIS-NECs originate from precursor lesions that usually give rise to non-neuroendocrine carcinomas of the corresponding organ, as evidenced by the detection of organ-specific genetic events (such as *KRAS* in pancreatic NECs, *APC* in colorectal NECs, and *ELF3* in ampullary NECs) [2]. Distinct genomic disparities were observed between pancreatic NECs (Panc-NECs) and non-pancreatic GIS-NECs (Nonpanc-NECs). The number of structural variations (SV) was significantly more notable in Nonpanc-NECs. Alterations in *TP53* and *RB1* were prevalent in GIS-NECs, while Nonpanc-NECs with intact Rb showed exclusive amplification of either *CCNE1* or *MYC*.

Abnormalities in the Notch family genes that originate from non-pancreatic digestive organs are observed in NECs. Therefore, the experiments utilized organoids that were derived from colonic and gastric mucosa. The organoid, which underwent genome editing in normal colon mucosa to delete *TP53* and *RB1*, exhibits minimal synaptophysin staining. This suggests that the deletion of *TP53* and *RB1* alone does not result in NEC. The administration of DAPT, a Notch inhibitor, leads to a significant increase in synaptophysin expression. The gastric organoid also yielded a similar observation. There is a notable upregulation of *PTF1A* and *RBPJL* in specific Panc-NECs, suggesting their derivation from pancreatic acinar cells.

In most GIS-NECs, there was an observed overexpression of transcription factors, notably the *SOX2* gene. The unexpected finding in this study was the high expression of *SOX2* in GIS-NECs, which seemed to be regulated by hypermethylation of the promoter region of *SOX2*, resulting in a phenomenon known as paradoxical gene activation. A notable positive correlation was observed between the methylation of *SOX2*'s promoter region and the expression of the gene. In order to further investigate this, we utilized ATAC-seq analysis. This technique helps map open chromatin structures throughout the genome by sequencing. The ATAC-seq analysis found that NECs with high SOX2 expression have open chromatin, which helps with gene transcription.

We have analyzed the epigenomic features of GIS-NEC and small cell lung carcinoma (SCLC) by combining motif enrichment analysis from ATAC-seq with enhancer profiling from a new CUT&Tag assay for H3K27ac [3]. We have found ELF3 to be a transcription factor connected to super-enhancers in NEC in cell lines affected by these diseases. By combining CUT&Tag and knockdown RNA sequencing methods, we unveiled the transcriptional network governed by ELF3 and identified its distinct gene signature including *AURKA*, *CDC25B*, *CLDN4*, *ITGB6*, and *YWAHB*. Furthermore, an assay investigating loss-of-function demonstrated that the depletion of ELF3 resulted in decreased cell viability. By analyzing gene expression in clinical samples, we effectively divided GIS-NEC patients into two separate groups using the ELF3 signature. Our research findings indicate that pathways promoting tumor growth were activated in the group exhibiting a high ELF3 signatur and provide insight into the transcriptional regulation of ELF3 as a cancer-causing factor in NEC [4].

A gastric NEC with Merkel cell polyomavirus (MCPyV) appeared to be caused by the monoclonal integration of MCPyV. The MCPyV large T antigen can directly bind to and deactivate *RB1*. A rectal NEC specimen showed intact Rb and p53, with no *CCNE1*/*MYC* amplification. It was determined that the sample tested positive for high-risk human papillomavirus (HPV-18). The results indicate that viral infection may be a contributing factor to Nonpanc-NECs.

Furthermore, there exists a potential for bidirectional development between NEC and Non-NEC components. The Rb protein was occasionally absent in both the NEC and Non-NEC components. Among these cases, it was observed that four driver mutations were present in both components, and oncogenic genes, including *KRAS* (p.G12A), exhibited an accumulation of driver mutations, specifically in the Non-NEC component. In addition, the evolutional lineage tree of our autopsied case indicated that the Non-NEC component (adenocarcinoma) was linked to a unique WGD (whole-genome duplication) -positive subclone.

These comprehensive studies of genomic alterations in GIS-NECs uncovered several key biological processes underlying the genesis of this very lethal form of cancer.

References

1. Yachida S, Totoki Y, Noë M, Nakatani Y, Horie M, Kawasaki K, Nakamura H, Saito-Adachi M, Suzuki M, Takai E, Hama N, Higuchi R, Hirono S, Shiba S, Kato M, Furukawa E, Arai Y, Rokutan H, Hashimoto T, Mitsunaga S, Kanda M, Tanaka H, Takata S, Shimomura A, Oshima M, Hackeng WM, Okumura T, Okano K, Yamamoto M, Yamaue H, Morizane C, Arihiro K, Furukawa T, Sato T, Kiyono T, Brosens LAA, Wood LD, Hruban RH, Shibata T. Comprehensive genomic profiling of neuroendocrine carcinomas of the gastrointestinal system. ***Cancer Discov***, 2022, 12, 692-711.
2. Yachida S, Wood LD, Suzuki M, Takai E, Totoki Y, Kato M, Luchini C, Arai Y, Nakamura H, Hama N, Elzawahry A, Hosoda F, Shirota T, Morimoto N, Hori K, Funazaki J, Tanaka H, Morizane C, Okusaka T, Nara S, Shimada K, Hiraoka N, Taniguchi H, Higuchi R, Oshima M, Okano K, Hirono S, Mizuma M, Arihiro K, Yamamoto M, Unno M, Yamaue H, Weiss MJ, Wolfgang CL, Furukawa T, Nakagama H, Vogelstein B, Kiyono T, Hruban RH, Shibata T. Genomic sequencing identifies *ELF3* as a driver of ampullary carcinoma. ***Cancer Cell***, 2016, 29, 229-240.
3. Horie M, Tanaka H, Suzuki M, Sato Y, Takata S, Takai E, Miyashita N, Saito A, Nakatani Y, Yachida S. An integrative epigenomic approach identifies *ELF3* as an oncogenic regulator in ASCL1-positive neuroendocrine carcinoma. ***Cancer Sci***, 2023, 114, 2596-2608.
4. Suzuki M, Saito-Adachi M, Arai Y, Fujiwara Y, Takai E, Shibata S, Seki M, Rokutan H, Maeda D, Horie M, Suzuki Y, Shibata T, Kiyono T, Yachida S. E74-like factor 3 is a key regulator of epithelial integrity and immune response genes in biliary tract cancer. ***Cancer Res***, 2021, 81, 489-500.

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THE ROLE OF BRCA1 TUMOR SUPPRESSOR IN *HELICOBACTER PYLORI*-MEDIATED GASTRIC CARCINOGENESIS

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Gastric cancer is the fourth most common cancer and the third leading cause of cancer-related deaths worldwide. Compelling evidence suggests that chronic infection with *Helicobacter pylori*, especially with *cagA*-positive strains, plays a vital role in the development of more than 80% of all human gastric cancer cases. Hence, *H. pylori* is the only known bacterium causally associated with human cancer. The *H. pylori cagA*-encoded CagA protein, produced inside the bacterium, is directly injected into the attached gastric epithelial cells via a bacterial microsyringe known as the type IV secretion system (T4SS) [1]. Once delivered into gastric epithelial cells, the *H.* bacterial CagA protein acts as a pro-oncogenic scaffold that promiscuously interacts with and functionally perturbs several host proteins, most notably the prooncogenic phosphatase SHP2 and polarity-regulating kinase PAR1b [2, 3, 4].

Although *H. pylori* infection is established in early childhood, gastric cancer generally develops in older adults, indicating that the direct oncogenic action of CagA does not work effectively at a younger age. Moreover, once established, gastric cancer cannot be cured by *H. pylori* eradication, indicating that *H. pylori*-driven gastric carcinogenesis proceeds through a "hit-and-run" mechanism, in which the maintenance of gastric cancer no longer requires *H. pylori* CagA [4].

Importantly, we and others recently found that the delivered CagA induces DNA double-strand breaks (DSBs) that underlie genomic instability in host gastric epithelial cells [5]. Mechanistically, the CagA-PAR1b interaction inhibits the kinase activity of PAR1b and thereby prevents the phosphorylation of the BRCA1 tumor suppressor, which is required for the cytoplasmic-to-nuclear translocation of BRCA1 to prevent fork stalling/collapse and subsequent DSB induction, while promoting error-free homologous recombination (HR)-

mediated DSB repair. Hence, CagA-delivered gastric epithelial cells undergo a status termed "BRCAness", characterized by excessive DSB formation that can only be repaired by error-prone non-HR mechanisms [5]. As long as the p53 tumor suppressor is functionally active in gastric epithelial cells, however, CagA-induced DSBs elicit premature cell senescence, which may promote the development of gastritis and ulceration in young adults infected with *cagA*-positive *H. pylori*. Functional inactivation of p53, mainly through aging-associated *TP53* mutations, allows for the expansion of cells with excess mutations by CagA-induced BRCAness, from which cancer precursor cells that no longer require cancer-promoting CagA activity may arise [5].

Indeed, sustained expression of CagA in gastric epithelial cells lacking *TP53* gives rise to a BRCAness-associated genome mutation signature characterized by SBS3 and ID6. The close connection between CagA and BRCAness was corroborated by a recent large-scale case-control study showing that the risk of gastric cancer in individuals carrying pathogenic variants of genes that induce BRCAness (such as *BRCA1*, *BRCA2*, *PALB2*, *and ATM*) increases dramatically upon infection with cagA-positive *H. pylori* [6].

In conclusion, our results indicate the following.

1) The CagA-PAR1b interaction induces BRCAness by inhibiting cytoplasmic-to-nuclear translocalization of BRCA1.
2) CagA-mediated BRCAness induces genomic instability, which causes mutations.
3) CagA-induced DSBs provoke p53-dependent G1 cell cycle arrest.
4) Loss of functional p53, most probably due to aging-associated TP53 mutation in normal gastric epithelial cells, cancels G1 arrest and thereby enables the expansion of CagA-expressing cells with genomic instability, from which CagA-independent cancer precursor cells that accomplish Hit-and-Run carcinogenesis may arise.
5) The presence of pathogenic BRCA variants dramatically increases the incidence in *H. pylori*-associated gastric cancer.

References

1. Murata-Kamiya N, Kikuchi K, Hayashi T, Higashi H, Hatakeyama M. *Helicobacter pylori* exploits host membrane phosphatidylserine for delivery, localization, and pathophysiological action of the CagA oncoprotein. Cell Host Microbe, 2010;7:399-411.
2. Higashi H, Tsutsumi R, Muto S, Sugiyama T, Azuma T, Asaka M, Hatakeyama M. SHP-2 tyrosine phosphatase as an intracellular target of *Helicobacter pylori* CagA protein. Science, 2002; 295:683-686.
3. Saadat I, Higashi H, Obuse C, Umeda M, Murata-Kamiya N, Saito Y, Lu H, Ohnishi N, Azuma T, Suzuki A, Ohno S, Hatakeyama M. *Helicobacter pylori* CagA targets PAR1/

MARK kinase to disrupt epithelial cell polarity. Nature, 2007;447:330-333.
4. Hatakeyama M. *Helicobacter pylori* CagA and gastric cancer: a paradigm for hit-and-run carcinogenesis. Cell Host Microbe, 2014;15:306-316.
5. Imai S, Ooki T, Murata-Kamiya N, Komura D, Tahmina K, Wu W, Takahashi-Kanemitsu A, Knight CK, Kunita A, Suzuki N, Del Valle AA, Tsuboi M, Hata M, Hayakawa Y, Ohnishi N, Ueda K, Fukayama M, Ushiku T, Ishikawa S, Hatakeyama M. *Helicobacter pylori* CagA elicits BRCAness to induce genome instability that may underlie bacterial gastric carcinogenesis. Cell Host Microbe, 2021;29:941-958.
6. Usui Y, Taniyama Y, Endo M, Koyanagi YN, Kasugai Y, Oze I, Ito H, Imoto I, Tanaka T, Tajika M, Niwa Y, Iwasaki Y, Aoi T, Hakozaki N, Takata S, Suzuki K, Terao C, Hatakeyama M, Hirata M, Sugano K, Yoshida T, Kamatani Y, Nakagawa H, Matsuda K, Murakami Y, Spurdle AB, Matsuo K, Momozawa Y. *Helicobacter pylori*, homologous-recombination genes, and gastric cancer. N Engl J Med, 2023;388:1181-1190.

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SOMATIC EVOLUTION, CANCER, AND OUR INEVITABLE DECLINE WITH AGE – INEXTRICABLY LINKED

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Abstract:

Why do we get cancer? Why is cancer highly associated with old age? Of course, aging is associated with the accumulation of more mutations, and some of these mutations can contribute to cancer phenotypes. But we now understand that carcinogenesis is much more complex than originally appreciated. In particular, there are tissue environmental forces that both impede and promote cancer evolution. Just as organismal evolution is known to be driven by environmental changes, cellular (somatic) evolution in our bodies is similarly driven by changes in tissue environments, whether caused by the normal process of aging, by lifestyle choices or by extrinsic exposures. Environmental change promotes selection for new phenotypes that are adaptive to the new context. In our tissues, aging or insult-driven alterations in tissues drives selection for adaptive mutations, and some of these mutations can confer malignant phenotypes (reviewed in (1)).

We have been using mouse models of cancer initiation, mathematical models of cellular evolution, and analyses of human tissue samples to better understand the evolutionary forces that control somatic cell evolution and thus cancer risk. We have shown that aging and inflammation dependent changes in stem cells and their tissue environments dramatically dictate whether cancer-causing mutations are advantageous to stem cells in our tissues, starting the cells down the path to cancer (e.g. (2-4)). Importantly, recent studies from many labs have shown how as we age our tissues become dominated by clones bearing mutations in known cancer-associated mutations (5, 6).

Cigarette smoking and old age are the strongest risk factors for the development of lung cancer. We analyzed mutational profiles from non-malignant biopsies of lung from multiple different cohorts [PEACE (NCT03004755), TRACERx (7)(NCT01888601) and CT-screened Colorado cohorts (8)]. For these analyses, we used the DuplexSeq method (9) and a ~50 kb panel covering the most commonly mutated regions in lung cancers, which allows for the detection of rare mutations with a sensitivity of less than 1 in 10^7. Using this method, we have identified large numbers of mutations in each brushing or biopsy, many of which are cataloged in the Catalogue Of Somatic Mutations In Cancer (COSMIC) (10), and most of these are predicted to disrupt protein function. We also observe pervasive positive selection acting on mutations in many but not all of the cancer-associated genes, with different patterns of selection in the lungs of ever-smokers and never-smokers. These clonal expansions not only provide potential initiating clones for lung cancer development, but are also predicted to perturb tissue function and integrity, as the genes mutated are known key players in cell specification, proliferation and function. Ongoing studies are using primary human and mouse lung epithelial cultures and mouse models to study how these mutations interact with smoking and age to alter associated clonal expansions, tissue integrity, and malignant progression. Our central hypothesis, to be further explored in the coming years, is that mutation-driven clonal expansions are promoted by both aging and cigarette smoking, that these clonal expansions can engender lung tissue decline, which in turn *further* promotes these clonal expansions. Thus, this feedforward loop can lead to accelerating lung dysfunction with age and exposures such as smoking. We further hypothesize that some (but not all) of these mutation-driven clonal expansions, together with the perturbations in the tissue microenvironment, can contribute to the evolution of lung cancers. Finally, we hypothesize that interventions that restore a healthier tissue microenvironment can interrupt this vicious cycle of clonal expansion and tissue decline, thus reducing disease risk.

In all, we propose a model whereby aging and contexts like smoking can promote selection for cells with adaptive mutations, which can contribute to not only cancer risk but also tissue aging. Thus, while young tissues impede selection for such adaptive mutations, old age or exposures such as from smoking are associated with a feed-forward loop of perturbed tissue-mediated selection for mutant clones that then increase tissue aging. Thus, understanding the forces controlling clonal selection as we age and due to lifetime exposures could be critical for controlling multiple diseases of old age.

References

1. Marongiu F, DeGregori J. The sculpting of somatic mutational landscapes by

evolutionary forces and their impacts on aging-related disease. Mol Oncol. 2022.
2. Henry CJ, Casas-Selves M, Kim J, Zaberezhnyy V, Aghili L, Daniel AE, et al. Aging-associated inflammation promotes selection for adaptive oncogenic events in B cell progenitors. The Journal of clinical investigation. 2015;125(12):4666-80.
3. Higa KC, Goodspeed A, Chavez JS, De Dominici M, Danis E, Zaberezhnyy V, et al. Chronic interleukin-1 exposure triggers selection for Cebpa-knockout multipotent hematopoietic progenitors. Journal of Experimental Medicine. 2021;218(6).
4. Rozhok A, DeGregori J. A generalized theory of age-dependent carcinogenesis. eLife. 2019;8:e39950.
5. Kakiuchi N, Ogawa S. Clonal expansion in non-cancer tissues. Nature reviews Cancer. 2021.
6. Evans EJ, Jr., DeGregori J. Cells with Cancer-associated Mutations Overtake Our Tissues as We Age. Aging Cancer. 2021;2(3):82-97.
7. Al Bakir M, Huebner A, Martínez-Ruiz C, Grigoriadis K, Watkins TBK, Pich O, et al. The evolution of non-small cell lung cancer metastases in TRACERx. Nature. 2023;616(7957):534-42.
8. Assante A, Lkhagvadorj K, Clambey ET, Danhorn T, Merrick DT, Keith RL, et al. Reduced Progenitor Function and Altered Immune Landscape Contribute to Field Cancerization of Lung Adenocarcinoma. Am J Respir Crit Care Med. 2023;208(8):903-5.
9. Kennedy SR, Schmitt MW, Fox EJ, Kohrn BF, Salk JJ, Ahn EH, et al. Detecting ultralow-frequency mutations by Duplex Sequencing. Nature protocols. 2014;9(11):2586-606.
10. Forbes SA, Tang G, Bindal N, Bamford S, Dawson E, Cole C, et al. COSMIC (the Catalogue of Somatic Mutations in Cancer): a resource to investigate acquired mutations in human cancer. Nucleic Acids Res. 2010;38(Database issue):D652-7.

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USING ULTRA-SENSITIVE SEQUENCING TO STUDY CLONAL EVOLUTION AND CANCER RISK

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Cancer evolves through mutation, selection, and clonal expansions. However, clonal expansions driven by cancer mutations are not exclusive of cancer: they also occur in histologically normal tissue during normal aging[1, 2]. Little is known about this process in part due to the difficulty to detect low frequency mutations in histologically normal tissue. Our group pioneered the use of ultra-sensitive duplex sequencing[3] to interrogate cancer driver mutations in non-cancerous human samples. We discovered prevalent *TP53* somatic evolution in individuals with and without cancer[4–6] and recently observed that an excess of *TP53* and *KRAS* clonal expansions in the normal colon may be associated with early-onset colorectal cancer[7]. Here, I will present novel data using ultra-sensitive duplex sequencing to characterize the landscape of clonal evolution in normal bladder during human lifespan.

We analyzed normal bladder urothelium collected via urothelial brushing at the top of the bladder (dome) and bottom of the bladder (trigone) from 26 individuals (16 males and 10 females), ages 24 through 86, who underwent autopsy at the University of Washington. We used ultra-deep duplex sequencing (~6,000x) to perform high sensitivity mutation detection in the most commonly mutated genes in normal urothelium and bladder cancer (16 genes). Sequencing reads were processed with in-house pipelines and mutations were characterized by gene and patient, compared to COSMIC, and analyzed for positive selection and mutational signatures.

Hundreds of mutations were identified in most samples, with some genes more frequently mutated than others, in close similarity to prior studies. The overall mutation

frequency increased with age, both in the dome and trigone samples, and the mutational landscape of both sample types was remarkably similar for most patients. The location of mutations within genes closely resembled the location of mutations reported for bladder cancer in COSMIC, and analyses of selection based on functional impact showed positive selection for most genes. Mutational signatures revealed a predominance of age, APOBEC, and smoking related signatures showcasing the contribution of these mutational processes to bladder clonal evolution.

Our results provide a novel, ultra-high resolution picture of the mutational landscape of the normal human bladder which, remarkably, was obtained by ultra-deep sequencing of single biopsies demonstrating the feasibility of this high-throughput approach for the study of somatic evolution at large scale. We confirm that clonal evolution is a prevalent process through human life and highlight the need to understand the promoting factors that enable the progression of initiated clones into cancer.

References

1. Kennedy SR, Zhang Y, Risques RA. Cancer-Associated Mutations but No Cancer: Insights into the Early Steps of Carcinogenesis and Implications for Early Cancer Detection. Trends Cancer 2019;5:531–540.
2. Risques RA, Kennedy SR. Aging and the rise of somatic cancer-associated mutations in normal tissues. PLoS Genet 2018;14:e1007108.
3. Kennedy SR, Schmitt MW, Fox EJ, et al. Detecting ultralow-frequency mutations by Duplex Sequencing. Nat Protoc 2014;9:2586–2606.
4. Krimmel JD, Schmitt MW, Harrell MI, et al. Ultra-deep sequencing detects ovarian cancer cells in peritoneal fluid and reveals somatic TP53 mutations in noncancerous tissues. Proc Natl Acad Sci U A 2016;113:6005–10.
5. Krimmel-Morrison JD, Ghezelayagh TS, Lian S, et al. Characterization of TP53 mutations in Pap test DNA of women with and without serous ovarian carcinoma. Gynecol Oncol 2019.
6. Salk JJ, Loubet-Senear K, Maritschnegg E, et al. Ultra-Sensitive TP53 Sequencing for Cancer Detection Reveals Progressive Clonal Selection in Normal Tissue over a Century of Human Lifespan. Cell Rep 2019;28:132-144 e3.
7. Matas J, Kohrn B, Fredrickson J, et al. Colorectal Cancer Is Associated with the Presence of Cancer Driver Mutations in Normal Colon. Cancer Res 2022;82:1492–1502.

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UNDERSTANDING OF GI CANCER INITIATION AND PROGRESSION USING ORGANOIDS

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In homeostatic adult tissues, niche factors play a crucial role in governing the long-term self-renewal and diverse differentiation potential of tissue stem cells. By recapitulating these niche factors in an in vitro environment, tissue stem cells have demonstrated the capacity to assemble into stereotypic organoid structures, sustaining long-term self-renewal. Notably, a spectrum of tissue-specific niche factors has been identified by our team and others, facilitating the successful propagation of organoids derived from various adult tissues. Human tissue-derived organoids have exhibited the remarkable ability to retain the genetic and epigenetic alterations inherent in the original tissues. Moreover, these organoids have shown disease-relevant biological characteristics both in vitro and in vivo.

Our comprehensive phenotypic analysis of patient-derived organoids has unveiled intricate molecular mechanisms governing genotype-phenotype correlations in human digestive tissue cancers. In parallel, the insights gleaned from genotype-phenotype correlations are harnessed in reverse through genome editing technology. The strategic introduction of genetic mutations into normal organoids has enabled the faithful recapitulation of tumor phenotypes. In this symposium, we will present our recent research showcasing genotype-phenotype associations in patient-derived organoids and engineered counterparts.

References

1. Ohta Y, Fujii M, et al., Sato T*, Cell-matrix interface regulates dormancy in human colon cancer stem cells. **Nature**. 2022; 608:784-794.

2. Sugimoto S, Kobayashi E*, et al., Sato T. * An organoid-based organ-repurposing approach to treat short bowel syndrome. **Nature**. 2021;592:99-104.
3. Kawasaki K, et al, Sato T*. An Organoid Biobank of Neuroendocrine Neoplasms Enables Genotype-Phenotype Mapping. **Cell**. 2020; 183: 1420-1435.
4. Nanki K, Fujii M, et al., Sato T*. Somatic inflammatory gene mutations in human ulcerative colitis epithelium. **Nature**. 2020;577:254-259.
5. Nanki K, Toshimitsu K, et al., Sato T*. Divergent Routes toward Wnt and R-spondin Niche Independency during Human Gastric Carcinogenesis. **Cell**. 2018;174:856-869.
6. Shimokawa M, Ohta Y, et al., Sato T*. Visualization and targeting of LGR5+ human colon cancer stem cells. **Nature** 2017;545:187-192.

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DECODING NORMAL BREAST TISSUE BIOLOGY AND THE EVOLUTION OF PREMALIGNANT BREAST CANCER

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To understand the transition from normal breast tissues to premalignant breast cancer, we must first understand the variation of cell types across women from different backgrounds and biological variables. To achieve this goal, we have led the Human Breast Cell Atlas (HBCA) that has analyzed normal breast tissues from over 126 women using single cell transcriptomics and spatial genomic approaches (Kumar et al., 2023, *Nature*). Our single-cell transcriptomics data profiled 714,331 cells and identified 12 major cell types and 58 biological cell states. These data revealed abundant pericyte, endothelial and immune cell populations, and highly diverse luminal epithelial cell states. Spatial mapping using four different technologies revealed an unexpectedly rich ecosystem of tissue-resident immune cells, as well as distinct molecular differences between ductal and lobular regions. Collectively, these data provide an unprecedented reference of the adult normal breast tissue for studying mammary biology and diseases such as breast cancer.

Ductal Carcinoma in Situ (DCIS) is the most common form of premalignant breast cancer and is often detected during annual mammography. DCIS presents a major clinical challenge for treatment decisions, since only about 10-30% of women recur with invasive disease within 10 years. This has led to significant over-treatment in many women with DCIS that may never recur with invasive breast cancer, who could have avoided the side-effects of therapy and elected 'active surveillance' with no treatment. However currently, there are no effective biomarkers that can be used to stratify DCIS patients into low-risk and high-risk groups. Here, we developed an archival single cell DNA-seq method (arc-well) and applied it to study FFPE tissues of DCIS samples and matched recurrences that occurred years to decades later (Wang et al., 2023, *Cell*).

Analysis of 10 patients with matched DCIS and cancers that recurred 2-16 years later showed that many primary DCIS had already undergone whole-genome-doubling, clonal diversification and shared genomic lineages with persistent subclones in the recurrences. Evolutionary analysis suggests that most DCIS cases underwent an evolutionary bottleneck, revealing chromosome aberrations in the persistent subclones that are associated with recurrence. In future work, we plan to use scRNA-seq and spatial transcriptomics to also understand the co-evolution of the TME together with the genetic changes which will undoubtedly lead to great insights into our understanding of breast cancer progression.

References

1. Kumar, T., Nee, K., Wei, R., He, S., Nguyen, Q.H., Bai, S., Blake, K., Pein, M., Gong, Y., Sei, E., et al. (2023). A spatially resolved single-cell genomic atlas of the adult human breast. *Nature*. 10.1038/s41586-023-06252-9.
2. Wang, K., Kumar, T., Wang, J., Minussi, D.C., Sei, E., Li, J., Tran, T.M., Thennavan, A., Hu, M., Casasent, A.K., et al. (2023). Archival single-cell genomics reveals persistent subclones during DCIS progression. *Cell*. 10.1016/j.cell.2023.07.024.

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LIFE HISTORIES OF BREAST CANCER AND RELATED CLONES

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Recent studies have documented frequent evolution of clones carrying common cancer mutations in apparently normal tissues, which are implicated in cancer development. However, our knowledge is still missing regarding what additional driver events take place in what order, before one or more of these clones in normal tissues ultimately evolve to cancer. Here, using phylogenetic analyses of multiple microdissected samples from both cancer and non-cancer lesions, we show unique evolutionary histories of breast cancers harbouring der(1;16), a common driver alteration found in approximately 20% of breast cancers. The approximate timing of early evolutionary events was estimated from the mutation rate measured in normal epithelial cells. In der(1;16)(+) cancers, the derivative chromosome was acquired from early puberty to late adolescence, followed by the emergence of a common ancestor by the patient's early 30s, from which both cancer and non-cancer clones evolved. Replacing the pre-existing mammary epithelium in the following years, these clones occupied a large area within the premenopausal breast tissues by the time of cancer diagnosis. Unexpectedly, evolution of multiple independent cancer founders from the non-cancer ancestors was common, contributing to intratumour heterogeneity. Our findings provide new insight into the evolution of breast cancer.

References

1. Nishimura T, Kakiuchi N, Yoshida K, Sakurai T, Kataoka TR, Kondoh E, Chigusa Y, Kawai M, Sawada M, Inoue T, Takeuchi Y, Maeda H, Baba S, Shiozawa Y, Saiki R, Nakagawa MM, Nannya Y, Ochi Y, Hirano T, Nakagawa T, Inagaki-Kawata Y, Aoki K,

Hirata M, Nanki K, Matano M, Saito M, Suzuki E, Takada M, Kawashima M, Kawaguchi K, Chiba K, Shiraishi Y, Takita J, Miyano S, Mandai M, Sato T, Takeuchi K, Haga H, Toi M, Ogawa S. Evolutionary histories of breast cancer and related clones. ***Nature***;620(7974):607-614, 2023.

2. Kakiuchi N, Ogawa S. Clonal expansion in non-cancer tissues. ***Nat Rev Cancer***;21(4):239-256, 2021.
3. Yokoyama A, Kakiuchi N, Yoshizato T, Nannya Y, Suzuki H, Takeuchi Y, Shiozawa Y, Sato Y, Aoki K, Kim SK, Fujii Y, Yoshida K, Kataoka K, Nakagawa MM, Inoue Y, Hirano T, Shiraishi Y, Chiba K, Tanaka H, Sanada M, Nishikawa Y, Amanuma Y, Ohashi S, Aoyama I, Horimatsu T, Miyamoto S, Tsunoda S, Sakai Y, Narahara M, Brown JB, Sato Y, Sawada G, Mimori K, Minamiguchi S, Haga H, Seno H, Miyano S, Makishima H, Muto M, Ogawa S. Age-related remodelling of oesophageal epithelia by mutated cancer drivers. ***Nature***;565(7739):312-317, 2019.

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CONCLUDING REMARKS

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In 2000, Hanahan and Weinberg made a marked impact on the field of cancer research with the publication of their seminal paper "The Hallmarks of Cancer". This review described cancer from the point of view of a complex tissue and enumerated biological capabilities that they argued were shared by virtually all cancers. Douglas Hanahan was invited to the 35th International Symposium of the Princess Takamatsu Cancer Research Fund, which was held in 2004, and Robert Weinberg was invited to the 37th International Symposium of the Princess Takamatsu Cancer Research Fund, which was held in 2006. Since then, tremendous progress has been made in our understanding of the development of cancers. This has forged remarkable advances in the early detection and treatment of several types of cancers. However, while the detection and treatment of early stage cancers is highly successful and treatment of advanced cancers that have metastasized to other tissues has improved, the 5-year survival rate of patients with advanced cancers remains poor. This emphasizes the continued critical importance of foundations such as the Princess Takamatsu Cancer Research Fund.

The Keynote Lecture of the 51st International Symposium of the Princess Takamatsu Cancer Research Fund was given by Curtis C. Harris. This Symposium examined several aspects of carcinogenesis, ranging from identification of environmental carcinogens to tumor promotion, DNA mutational signatures, and the multiomics of precision medicine, and included the association of the microbiome with different cancers.

It is well known that limiting exposure to environmental and workplace carcinogens is of the utmost importance in cancer prevention. Two speakers discussed the use of animal models to identify human carcinogens. Work with humanized-liver mice found that

acetoaceto-o-toluidide (AAOT) is metabolized to o-toluidine (OTD) by humanized-liver cells, and since OTD is a known human bladder carcinogen, the presence of OTD in the bladder of these mice identifies AAOT as a probable human bladder carcinogen. Another study using intratracheal instillation, a method known as TIPS, found that all tested carbon nanotubes were carcinogenic to the rat lung. Implementation of regulations for the safe manufacture and use of these materials could have a tremendous impact on human cancers.

Another topic of this symposium concerned the pathways by which carcinogens induce cancer. Prostate cancer is the fifth most lethal cancer for males. A talk on prostate cancer described how many environmental toxins target prostate stem-progenitor cells, and due to the long-lived nature of these cells, the effects of these toxins increase prostate carcinogenic potential over extended time periods. Gastric cancer is the fourth leading cause of cancer mortality. *Helicobacter pylori* infection is a known risk factor for gastric cancer. Up to 96% of *H. pylori* express CagA, which interacts with PAR1b and inhibits the kinase activity of PAR1b. This prevents phosphorylation and activation of BRCA1, resulting in an increase in DNA double strand breaks and an increase in mutation levels. p53 protects against CagA induced stomach cancer. As people age, they have decreased p53, and consequently, young people infected with *H. pylori* often develop gastric cancer as they age. Notably, these pathways not only increase our understanding of carcinogenesis, but can be used clinically. *H. pylori* infection also induces inflammation and the methylation level of a single marker gene, *miR124a-3*, accurately predicts gastric cancer risk. Another pathway that was shown to have probable clinical utility was the histone H3.3 specific chaperone HIRA and its target histone H3.3. This pathway is a promising target to prevent cancer progression.

Another major topic of this symposium was the clinical use of mutational signatures. Mutational signatures are patterns of mutations characteristic of exposure to different carcinogens. The Nakahara Memorial Lecture was delivered by Arthur P. Grollman. In this lecture he discussed the mutational signature of aristolochic acid (AA), a powerful nephrotoxin and human carcinogen. This mutational signature has been detected in several different cancer types and in healthy tissue, identifying persons exposed to AA in the absence of tumor development. AA is bioactivated in the liver and active AA metabolites complex with human serum albumin in the circulatory system. These complexes are transported to a variety of tissues where they elicit mutational effects. Dr. Grollman then described the development of a test known as UroSEEK which surveys mutations and copy number changes and is able to detect urothelial cancer cells shed into the urine, allowing early detection of urothelial cancers.

Technology that identifies patterns of DNA adducts, which can result in contextual mutational signatures, was also described. Knowledge of DNA adduct and DNA mutational profiles allows identification of driver adducts and driver mutations associated with

carcinogenesis, and this significantly advances our knowledge of the events that cause cancer. Advancements in metabolic profiling and technology able to detect all DNA adducts induced by exposure to various chemicals allows characterization the effect of specific chemicals and their metabolites on DNA and has important implications for genotoxicity testing, biomonitoring, and improved cancer prevention and treatment. Use of mutational signatures and DNA adductomics to improve detection and therapy of cancer in patients with gastric cancer, liver cancer, and renal cancer was described. The DNA landscape of driver events in gastric cancer allows identification of individuals at risk of developing gastric cancer. This in turn allows extensive endoscopic screening of these patients and application of preventive interventions. For liver cancer, the third most lethal cancer, adductomics was used to identify risk factors. Finally, DNA mutation profiles associated with renal cancer indicated the existence of multiple, geographically variable, mutagenic exposures and promises to expand our knowledge of the causes of renal cancer. In addition, the use of mutational signatures to uncover an inflammatory pathway driven by IL1B associated with non-small cell lung cancer in never smokers induced by exposure to air pollution particulate matter was discussed.

Epidrivers can be considered a type of driver mutation signature. The epigenome consists of chemical compounds that modify, or mark, the genome and affect its function, for example, DNA methylation. Genes encoding proteins that directly regulate the epigenome are known as epigenetic regulator genes (ERGs). Analysis of ERGs in 11,000 tumor and normal tissue specimens identified a subset of ERGs that were altered at the genetic or RNA expression level across many malignancies. These epidrivers confer traits associated with the hallmarks of cancer onto cells, indicating that epidrivers can act as drivers of tumorigenesis. DNA methylation is part of the epigenome, and as noted above, the methylation level of a single marker gene, *miR124a-3*, accurately predicts gastric cancer risk. Neuroendocrine carcinoma of the gastrointestinal system (GIS-NEC) is a rare but highly malignant cancer with extremely poor patient outcomes. A talk describing comprehensive studies of the alterations in GIS-NECs noted that epigenetic aberrations play a significant role in GIS-NECs, especially hypermethylation of the promoter region of SOX2 resulting in high expression of SOX2 in most GIS-NECs.

Driver mutations can occur years, even decades, before events that promote the development of cancer. Mouse skin tumors were induced in different mouse models and at different times in the life of the mice by exposure to different regimens of mutagens followed by exposure to strong inflammatory processes. Whole genome sequencing of over 100 of the tumors revealed that cells with mutations sufficient to initiate carcinogenesis could remain in the skin for an extended period of the life of the mouse until activated by a promoting factor. The promoting factors did not necessarily cause mutations. This suggests the probable

existence of unidentified factors that promote human cancers. Another talk discussed the use of ultra-sensitive duplex DNA sequencing to characterize clonal evolution in normal human bladder urothelium. Clonal evolution is a process that occurs throughout human life, and mutation frequency increased with age. Mutational signatures in normal urothelium often resembled those found in bladder cancers, highlighting the need to understand promoting factors and the mechanism by which they enable the progression of initiated clones into cancer. Another talk discussed age and changes in the tissue environment, and how these changes drive selection of cells with adaptive mutations, thus driving clonal evolution as we age. The talk concluded that young tissues and old tissues respond differently to the tissue environment, and a tissue environment that doesn't promote tumorigenesis in young tissue may promote tumorigenesis in old tissue. Overall, these talks highlighted the need to understand the exact mechanism of tumor promotion.

In agreement with the conclusion that driver mutations can occur many years prior to tumor promotion, phylogenetic analysis of microdissected samples from cancer and non-cancer lesions in the breast identified driver mutations that were acquired in early puberty to late adolescence from which both cancer and non-cancer clones evolved. These clones occupied a large area within the premenopausal breast tissue by the time of cancer diagnosis. Evolution of multiple independent cancer founders from non-cancer ancestors was common, resulting in intratumor heterogeneity, suggesting a role for epigenetic changes and/or the microenvironment in cancer development. As noted above, the microenvironment is a potential non-mutagenic promoter of cancer. Another potential non-mutagenic cancer promoter is obesity, which was the topic of another talk. Metabolomic and proteomic studies have the potential to unravel the obesity-cancer link, enabling informed preventative strategies and the formulations of effective public health policies, as well as enriching our understanding of the mechanisms of tumor promotion.

It is well known that the gut microbiota impacts colorectal cancer (CRC). One talk discussed the relationship between specific bacteria and their communities and CRC. While challenging, it was proposed that translational research on the manipulation of the microbiota for the therapeutic benefit of cancer patients is feasible. Another talk discussed the effect of spinach on the microbiome. In a rat model, spinach increased microbiome diversity and exhibited significant antitumor activity. It was determined that the antitumor activity was linked to spinach derived linoleate bioactives and altered butanoate metabolism arising from increased alpha-diversity of the gut microbiome. Linoleate and butanoate metabolites inhibited histone deacetylase (HDAC), induced apoptosis, and altered IFN-gamma signaling in human and mouse colon cancer cells. In addition, IL-2 secretion was increased in T-cell hybridomas co-cultured with the colon cancer cells. Thus, therapeutic targeting of the microbiome is feasible. Another talk discussed the relationship of oral

microbiome dysbiosis with orodigestive cancer, including pancreatic cancer, in humans. Cancer-associated oral microbiota play a pivotal role in metabolic production of carcinogens. In addition, the gut microbiome was shown to predict therapy response in non-small cell lung cancer patients and melanoma patients. In addition, another talk discussed the utility of the Collaborative Cross mouse as an optimal model for deciphering gene-environment-microbiome interactions in carcinogenesis. This mouse can be used to develop novel therapies, including microbiome-targeted therapies, for the prevention and treatment of cancer.

Two talks discussed the use of *in vitro* testing systems. One talk discussed determining the mutational signatures of test agents. It was determined that the mutational signatures of 4 different carcinogens were qualitatively similar in human tissue organoids derived from the stomach, colon, liver, pancreas, and kidney. The mutational signatures of an additional 16 environmental carcinogens were investigated in human gastric organoids, and 79 agents were tested using induced human pluripotent stem cells (hIPSCs). The 16 carcinogens produced 12 different signatures in the organoids and the 79 agents produced 41 different signatures in hIPSCs, providing insights into processes underlying the causes of human cancer. The other talk discussed the use of patient derived organoids to examine genotype-phenotype associations in human digestive tissue cancers. This technique allows development of therapeutic strategies to combat cancer.

Two talks discussed the use of aspirin for cancer prevention. In young people, but not older people, aspirin decreases prostaglandin metabolites in the urine, deceasing inflammation and cancer; aspirin also decreases kynurenate and colorectal cancer; and aspirin also decreases hepatic fat and liver cancer. The other talk described the use of enteric-coated aspirin (100 mg/day). In a clinical trial it was found that 100 mg/day enteric-coated aspirin decreased the recurrence of CRC. In a second clinical trial, aspirin inhibited the growth of colorectal polyps of more than 5 mm in diameter in patients with familial adenomatous polyposis.

One of the final talks of the symposium described the development of a single-cell DNA sequencing method that could be used with archived formalin-fixed paraffin-embedded tissue samples from patients with Ductal Carcinoma in Situ (DCIS). Analysis of the genome profile of 10 patients with DCIS and breast cancers that recurred 2-16 years later enabled the identification of chromosome aberrations that were associated with recurrence. Since only about 10-30% of women with DCIS develop invasive breast cancer, this DNA sequencing method can be used to identify women at high-risk of developing invasive breast cancer and women with DCIS that may never develop invasive breast cancer, allowing treatment of the women at high-risk and allowing women at low-risk to choose either therapy or active surveillance with no treatment to avoid the side-effects of therapy.

The 51st International Symposium of the Princess Takamatsu Cancer Research Fund demonstrated several advancements in our understanding of the mechanisms of carcinogenesis and the detection and treatment of cancer. Advanced, metastatic cancers still presents a tremendous challenge, and the 5-year survival rate of patients with advanced cancers remains poor. We look forward to the 52nd International Symposium of the Princess Takamatsu Cancer Research Fund and the further advancement of cancer therapies.

I would like to express my sincere gratitude to the organizers of this symposium, Drs. Allan Balmain, Hitoshi Nakagama, Fuyuki Ishikawa, and Yukari Totsuka. I would also like to express my sincere gratitude to the secretary general of the symposium Dr. Michihiro Mutoh. I would also like thank the speakers and all of the participants for the excellent presentations and fruitful discussions, which greatly contributed to the success of the symposium. Finally, I would also like to express my deep gratitude to the the Foundation for its support and the work that was put into this symposium.