Title

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Author

Carethers, John

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THE JEREMIAH METZGER LECTURE: ENVIRONMENTAL INFLUENCES ON COLORECTAL CANCER

JOHN M. CARETHERS, MD

SAN DIEGO, CALIFORNIA

ABSTRACT

Gene-environmental interactions create risk profiles for sporadic cancer development in patients with colorectal cancer (CRC). For instance, a person's socioeconomic status over their lifetime can affect their level of physical activity and type of diet, and their exposure to tobacco and alcohol may affect their gut microbiome and ultimate risk for developing CRC. Metabolic disease can independently or further change the gut microbiome and alter the typical timing of CRC development, such as is observed and linked with early-onset disease. Patients with microsatellite unstable tumors where DNA mismatch repair is defective have altered immune environments as a result of tumor hypermutability and neoantigen generation, allowing for immune checkpoint inhibitor susceptibility; in such cases, the genetics of the tumor changed the environment. The environment can also change the genetics, where interleukin-6-generated inflammation can inactivate MSH3 protein function that is associated with CRCs which are more metastatic, and patients show poor outcomes. Some specific aspects of the local microbial environment that may be influenced by diet and metabolism are associated with CRC risk, such as *Fusobacterium nucleatum* infection, and may affect the initiation, perpetuation, and spread of CRC. Overall, both the macro- and microenvironments associated with a person play a major role in CRC formation, progression, and metastases.

ENVIRONMENTAL EPIDEMIOLOGY AND COLORECTAL CANCER

Colorectal cancer is a genetic disease whose initiation, progression, and potential spread beyond the colon are influenced by the

Correspondence and reprint requests: John M. Carethers, MD, Division of Gastroenterology and Hepatology, Department of Medicine, University of California San Diego, San Diego, CA, Moores Cancer Center, University of California San Diego, San Diego, CA, Herbert Wertheim School of Public Health and Human Longevity Science, University of California San Diego, San Diego, CA, 9500 Gilman Drive, La Jolla, CA 92093, Tel: 858-534-1501, E-mail: jcarethers@health.ucsd.edu.

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macroenvironment of the individual and the microenvironment of the colon. Histologically, most CRCs develop from precursor adenomatous polyps which are initiated genetically through faulty Wnt signaling usually through acquired somatic *APC* gene mutation. Adenomas can progress in size in conjunction with acquisition of activating mutations in the proto-oncogene *KRAS* and loss of canonical SMAD signaling, and after a period of genomic chaos, acquire *TP53* mutations to become carcinomas (1). As the adenoma may enlarge to become cancer and continue to grow, it often sheds blood; thus, screening programs utilizing stool-based tests as well as endoscopic visualization or novel blood-based tests can identify these tumors (2).

The majority (about 65%) of CRCs develop sporadically, meaning there is lack of any CRC in the past two to three generations of an individual's family. The remaining 35% of persons with CRCs have evidence for a familial component, but only about 5% of the total CRCs with a familial component have a mono-allelic, mendelian-inherited, high-risk gene identified, with the most common being Lynch syndrome [inherited mutation in the DNA mismatch repair (MMR) genes] and familial adenomatous polyposis (inherited mutation in the *APC* gene), plus some uncommon inherited hamartomatous polyposis syndromes (3). To understand what the more important contributor toward the development of sporadic cancer might be—heredity or the environment—Lichtenstein et al assessed nearly 45,000 twins of which nearly 10,000 individuals developed cancer from 1900 to 1990 and used mathematical models to assess the relative contribution associated with cancer development (4). Only three cancers among 28 sites showed a statistical heritable component—prostate at 42%, CRC at 35%, and breast at 28%—which contributed to the association with cancer, whereas all cancers were linked with the environment (4). For CRC, 65% of its contribution was from the environment (4). These authors concluded that the environment plays a dominant role in causing sporadic cancer among humans (4). In addition, multiple examples of environmental-gene interactions are associated with cancer development in humans. In essence, with nearly 8 billion unique individuals in the world, all with varied risk based on their background and genetic makeup, exposure to something in the environment influences and extends cancer risk from that baseline genetic risk (Figure 1). For example, tobacco usage greatly increases one's cancer risk at multiple sites, but not everyone who uses tobacco develops a cancer.

Risk factors for CRC can be separated into non-modifiable risk factors and modifiable risk factors that combine to confer lifetime risk for CRC (Figure 2). Non-modifiable risk factors are ones an individual

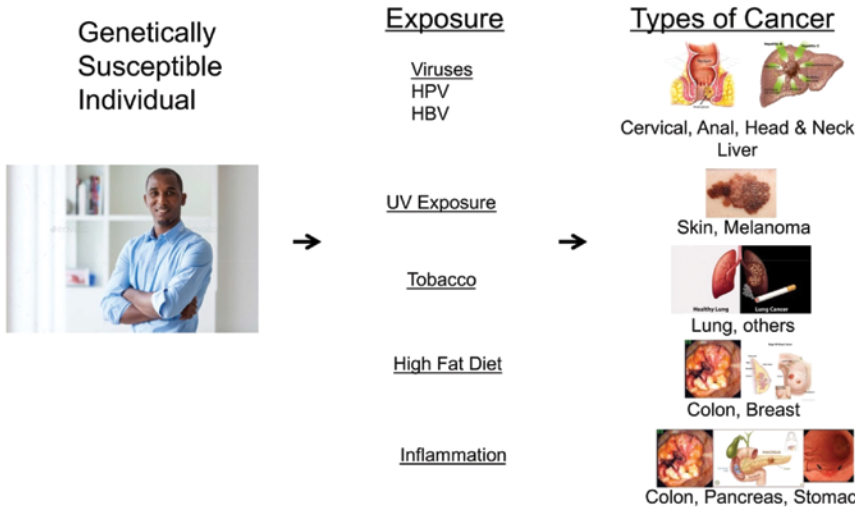

FIG. 1. Examples of Gene-Environmental Interactions for Cancer Development in Humans. Each human has a different level of genetic susceptibility based on their genetic makeup and, therefore, has a variable risk for developing a cancer if exposed to a pro-carcinogenic environmental agent.

is born with or cannot modify and include high-risk (e.g., a mutation in a DNA MMR gene) and low-risk alleles and overall genetic makeup (individual single nucleotide polymorphisms) that may confer polygenic risk, along with the individual's age (Figure 2). Modifiable risk factors are ones an individual or society can modify, such as an individual's diet, use of tobacco and nonsteroidal anti-inflammatory agents, hormone replacement therapy, and level of physical activity, along with CRC screening utilization (Figure 2). For instance, supplemental use of vitamin D and/or calcium can lower one's lifetime risk for CRC by about 10%. People in the United States who live north of the 37th parallel where the levels of sun exposure are lower have higher rates of CRC compared to the population south of the 37th parallel (5). The one exception to this observation is that south of the 37th parallel there are hotspots for CRC largely within the Mississippi River delta and Appalachia where there is poverty, high unemployment, low education, poor health access, and low CRC screening rates (6). Likewise, risk for CRC varies among races/ethnicities in the United States with the highest incidence and mortality occurring among Native Americans and non-Hispanic Blacks compared to non-Hispanic Whites, Asian Pacific Islanders, and Hispanic populations (6,7). The observation of an

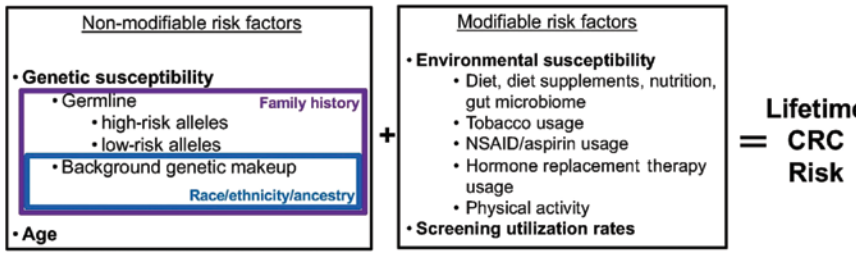

FIG. 2. Risk Factors for Colorectal Cancer. Ultimate lifetime risk for colorectal cancer is determined by non-modifiable factors such as age and genetic susceptibility, plus modifiable factors exposed over one's lifetime. Colorectal cancer screening can mitigate risk if utilized effectively.

incidence and mortality disparity extends to young adults 20-44 years of age with rates being higher for non-Hispanic Blacks compared to non-Hispanic Whites and Asian Pacific Islanders (no data available for Native Americans) (8,9). These data suggest that this disparity develops at an early age before CRC screening commences. The disparity likely stems from origins of socioeconomic inequality where populations, such as in Appalachia and the Mississippi River delta, have lower socioeconomic status, lower levels of education, and less access to health care (5,6). As a consequence, these populations reside in lower-income neighborhoods where there are grocery deserts and recreational park deserts, work several low-paying jobs to make ends meet, have poor access to and ability to afford healthy foods, engage in less physical activity, and use less preventive medicine. Over many years, this disparity creates metabolic consequences in which the gut microbiome is altered and localized gut inflammation increases. Biological consequences include increased colonic crypt proliferation and increased and earlier adenoma formation through somatic gene mutations (5,6). Thus, socioeconomic inequality is connected to an increase in CRC risk through these built-on relations, with ultimate biological effects (5,6). When African Americans and rural Africans exchange a Western diet (high fat, low fiber) for a low fat, high fiber African-style diet, within two weeks of the diet exchange, there is a significant effect on colonic epithelium growth characteristics (10). Rural Africans following the Western diet experienced doubled colonic crypt proliferation, significantly increased epithelial inflammation, higher pro-carcinogenic deoxycholic acid, and lower health-associated butyrate levels; reciprocal changes were observed in the African Americans following the African-style diet (10). These data show that dietary influence can

happen rapidly and directly affect metabolic products and epithelial growth. For instance, consuming a Western diet for decades will likely increase colonic epithelial proliferation and the chances for a somatic genetic event that can transform into neoplasia. This concept suggests direct environmental contributions to disparities observed for CRC incidence (5). When non-Hispanic Blacks are compared to non-Hispanic Whites, there is strong evidence for the presentation of (a) an increase in large and high-risk adenomas >9mm, (b) increase in proximal (right side of the colon) adenomas, (c) earlier onset for CRC, (d) increase in proximal CRCs, (e) increase in sulfidogenic bacteria in the colon, and (f) increase in pro-inflammatory *Fusobacterium* and *Enterobacter* species in the colon (5). Additional differences for non-Hispanic Blacks with CRC include decreased numbers of CD8+ T lymphocytes and granzyme B+ T lymphocytes within CRCs (11-14).

This CRC incidence and mortality disparity caused some professional organizations to call for commencing screening earlier for non-Hispanic Blacks than guidelines recommend (15). However, over the past three decades, the number of early-onset CRCs (CRCs in patients under the age of 50 years) increased from approximately six CRC cases per 100,000 population among non-Hispanic Whites and Hispanics, approximating the same percentages seen among non-Hispanic Blacks at 12 CRC cases per 100,000 population (16-18). Early-onset CRCs now make up 12% of all new cases of CRC, up from 5.5% during the 1990s (16,17). This increase prompted professional organizations as well as the U.S. Preventive Services Task Force to recommend that CRC screening commence at age 45 years for all Americans, regardless of race or ethnicity (16,17). Epidemiology of early-onset CRC appears to be driven by a birth cohort effect: those born before 1960 had ongoing extremely low rates for early-onset CRC, whereas those born after 1960 show increasing rates each year for early-onset CRC (16,19). The incidence per 100,000 population is highest for the age tier 45-49 years (~33/100,000), next for age tier 40-44 years (~19/100,000), etc. (16,19). For age tiers over 50 years, CRC incidence continues to fall presumably due to active CRC screening (16,19). Thus, the increase in early-onset CRC incidence appears to be driven by environmental factors and not inherited genetic factors. There are several examples of environmental changes that alter the risk for CRC (Table 1). Rapid increases or decreases in CRC risk are due to changes in the environment and not changes in genetics from the germline. In some examples listed in Table 1, individual genetic background in one environment may demonstrate low risk for CRC, but once the environment changes, the risk is altered dramatically. For early-onset CRC, two plausible hypotheses

TABLE 1

Examples of Environmental Change in Humans That Modify Colorectal Cancer Risk

Japanese immigrants to the West Coast of the United States pre-World War II
• Within two generations had similar colorectal cancer rates as other Americans
Export of fast food from the United States to Japan post-World War II
• Marked elevated colorectal cancer rates that exceeded American rates likely due to genetic predisposition but laced the dietary environmental influence previously
Middle class development in China post-2000
• Increase in metabolic syndrome, colorectal cancer, other cancers
Aspirin/nonsteroidal anti-inflammatory drugs
• Associated with up to 50% reduced colorectal cancer risk
Vitamin D and calcium
• Associated with up to 12% reduced colorectal cancer risk
Hormone replacement therapy
• Associated with moderate reduced colorectal cancer risk in women
Low fat, low caloric, low red meat diet
• Associated with moderate reduced colorectal cancer risk long term

from the environment may explain the increase in CRCs in individuals under 50 years old (20). First, after an environmental exposure to all persons, those over age 50 are screened showing a reduction in CRC prevalence, while those under age 50 have an apparent relative increase in CRC prevalence. Second, after an environmental exposure to all persons, the environmental exposure selectively affects younger individuals (who are not screened) resulting in a true increase in CRC prevalence (20). Early-onset CRCs are predominantly located in the rectosigmoid colon, which does not follow the pattern seen in young non-Hispanic Blacks where there is a predominance of proximal CRCs. The most common risk factors linked to early-onset CRC development are metabolic dysfunction, microbiome dysbiosis, and exposure factors (Table 2) (21). For instance, high fructose corn syrup use as a diet addition has skyrocketed over the past five to six decades and is thought to be a contributor toward the development of type 2 diabetes mellitus and fatty liver disease. Using *APC*-null mice, consumption of 20 g of high fructose corn syrup (for which 5 g overwhelms small intestine GLUT5 transport) daily for one month showed greatly enhanced intestinal tumor growth (in number and histological grade) over conventionally fed *APC*-null mice and was independent of obesity (22). There was notable uptake of fructose within the intestinal tumors associated with accelerated *de novo* fatty acid synthesis in the lesions (22). This

TABLE 2

Environmental Links to Increases in Early-Onset Colorectal Cancer

High caloric and high red meat (Western) diet
• May affect the gut microbiome and cause intestinal dysbiosis
Presence of metabolic disease such as type 2 diabetes
• May be related to hyperglycemia, insulin resistance, and/or hyperinsulinemia
• May be related to chronic inflammation and oxidative stress
Low level of physical activity over time (increased sedentary time)
Tobacco usage
Alcohol usage
Unknown direct carcinogen exposure

study implies metabolic acceleration of intestinal tumor growth due to ingestion of high fructose corn syrup. Based upon transcriptional differences between early- and later-onset CRC cases in databases, early-onset CRCs showed higher expression of ALDH1A1, a cancer stem cell marker involved in xenobiotic and retinoic acid metabolism, and immune modulation (23). CRC resections are increasing among adults undergoing surgery for obesity management and are declining among adults lacking obesity (24). An additional study suggests that type 2 diabetes contributes to the cumulative risk for CRC as much as the presence of family history of CRC, and both the presence of diabetes and a family history of CRC greatly lowered the predicted age of onset for CRC due to synergism (25). In total, the actual cause for the increase in early-onset colorectal cancer is not yet known but is driven by changes in environment, with strong association with metabolic disease. Despite this, it may be hard to tease out these drivers separate from later-onset CRC, as the listed risks are similar for CRC development regardless of age. The environmental exposure for early-onset CRC might affect tumor initiation (e.g., adenoma formation) but could also play a role in tumor progression and metastasis, all while the cancer accumulates genetic and epigenetic mutations (20). Several groups are investigating the cause and drivers of the increase of early-onset CRCs.

Environment Changing the Genetics of Colorectal Cancer

DNA damage occurs constantly to cells, driven by exposures from the local environment. These include oxidative damage, UV exposure, direct-acting carcinogens, cytosine deamination to uracil, and DNA

replication errors, among other mechanisms. The DNA damage leads to blocked DNA replication and transcription, genomic instability, and mutation. In a somatic cell that is terminally differentiated, the DNA damage may not matter as it undergoes cell demise; in a stem cell that is capable of self-renewal, DNA damage can be propagated and potentially transform the cell into cancer (26).

Over the past decade, an environmentally driven mechanism to inactivate DNA MMR was discovered and can worsen the outcome of patients whose CRCs developed from the chromosomal unstable, microsatellite unstable (MSI-H), or CpG island methylator phenotype pathways (1,27). About 50% of all CRCs manifest elevated microsatellite alterations at selected tetranucleotide repeats (EMAST), driven by dysfunction of the DNA MMR protein MSH3 (1,27-41). Features of EMAST CRCs include the lack of mononucleotide repeat instability (due to sole inactivation of MSH3 and not *MSH2*, *MLH1*, *MSH6*, and *PMS2*), possessing a non-hypermutated genome, and being intimately associated with inflammatory cells (Table 3). Patients possessing EMAST CRCs show worse outcomes over patients with MSI-H tumors or microsatellite stable tumors (28), and a higher proportion of EMAST CRCs are found in non-Hispanic Black patients compared to non-Hispanic White patients (Table 3) (33). Unlike that observed for MSI-H CRCs where germline or somatic mutation of *MSH2*, *MLH1*, *PMS2*, or *MSH6* is found, no MSH3 mutation was identified among EMAST CRCs. Instead, the associated inflammatory cells seen in EMAST CRCs, along with potential contributions from epithelium, drive loss-of-function of MSH3,

TABLE 3

Common Features of EMAST (Elevated Microsatellite Alterations at Selected Tetranucleotide Repeats) Colorectal Cancers and Patients

Tumors observed in up to 50% of all sporadic colorectal cancers.
Tumors display elevated microsatellite alterations at selected tetranucleotide repeats on PCR assay in absence of mononucleotide repeat alterations.
Tumors display heterogeneous (reduced) MSH3 protein expression.
Tumors appear distributed throughout the colon and not specific to one segment.
Tumors are non-hypermutated genetically.
Tumors are associated intimately with inflammatory cells.
Patient tumors are sensitive to 5-fluorouracil-based therapy.
Patient tumors are unlikely responsive to immune checkpoint therapy.
Patients with EMAST tumors show shorter survival, increased metastasis, poor prognosis, and advanced stated disease.
Patients of African descent in the United States experience higher frequency of rectal tumors.

rather than mutation of MSH3 (32-35,39-41). Locally released interleukin-6 (IL6) triggers oxidative stress, and IL6 directly shifts MSH3 from the nucleus to the cytosol where it cannot repair DNA (29-31). Importantly, other pro-inflammatory cytokines did not cause shifts other than IL6; likewise, the other DNA MMR proteins did not shift with IL6 other than MSH3 (29-31). Blockage of IL6 signaling down its JAK/STAT3 kinase pathway prevented the MSH3 nucleus-to-cytosol shift, clearly indicating dependency on this pathway (30). Within two weeks of IL6 exposure, cells accumulated a nearly 10-fold increase in intrinsic tetranucleotide frameshifts over non-IL6 treated cells (30,38). Unlike patients with MSI-H tumors that show resistance to 5-fluorouracil chemotherapy, patients with EMAST CRCs retain 5-fluorouracil susceptibility (Table 3) (37). Because of the association with inflammatory cells and IL6 production to trigger EMAST, EMAST may also be called "inflammation-associated microsatellite alterations" (41). The schema for development of EMAST CRCs starts with independent initiation of early neoplasia and tumor advancement but could be propagated by the onset of inflammation and IL6 release, perhaps from interaction with microbiota (32). Once IL6 is released, the epithelial cells have a shift of MSH3 protein from the nuclear to the cytosol compartment, abrogating MSH3's ability to repair DNA. The type of DNA damage with loss-of-function of MSH3 includes increases in di-, tri-, and tetra-nucleotide repeat instability, but also DNA double strand breaks due to some evidence that MSH3 is involved in homologous recombination repair (32,39-41). As a consequence, and perhaps more importantly driven by contributions from DNA double strand breaks, patient outcomes with EMAST CRCs show the biological consequences of increased metastasis and poor survival (32,41).

The outcomes of patients with EMAST tumors are in contrast to patients with MSI-H tumors (Figure 3). Patients with MSI-H CRCs have tumors that are hypermutated due to multiple single point mutations and frameshift mutations at coding microsatellites that generate immunogenic peptides. The neoantigens vaccinate the tumor to reduce metastases and improve survival compared to patients without MSI-H CRCs (1,36). Patients with EMAST tumors and MSH3 dysfunction do not generate mononucleotide instability and thus lack the generation of immunogenic peptides and do not create the conditions for "self-vaccination" and subsequent immunological containment of the tumor (32,39-41). Instead, sole MSH3 dysfunction creates conditions leading to higher metastases and poor survival (Figure 3). Overall, for EMAST CRCs, the local environment through IL6 release can cause genetic mutation via lack of MSH3's ability in DNA MMR and in homologous recombination repair.

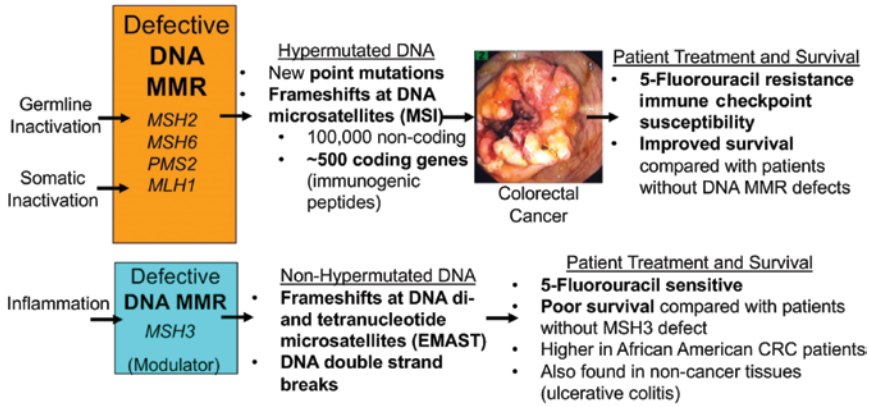

FIG. 3. Progression of DNA Mismatch (MMR) Repair Deficient Colorectal Cancers and Outcome Consequences. **(Top)** Inactivation of *MSH2*, *PMS2*, *MSH6*, and *MLH1* either through germline or through somatic mechanisms leads to tumor microsatellite instability-high (MSI-H) and hypermutated DNA. Because of a limited number of coding (mostly mononucleotide) microsatellites, MSI-H tumors generate novel peptides that are immunogenic, and along with their hypermutability provide responsiveness to immune checkpoint inhibitor drug susceptibility. MSI-H tumors are relatively drug resistant to 5-fluorouracil therapy, but patients with MSI-H tumors have improved survival compared to patients without MSI-H tumors. **(Bottom)** Interleukin-6-driven inflammation can somatically inactivate MSH3 by moving it from the nucleus to the cytosol. This causes a unique form of microsatellite instability called EMAST (elevated microsatellite alterations at selected tetranucleotide repeats) that does not involve mononucleotide instability. MSH3 is also involved in homologous recombination events that in its absence can lead to DNA double strand breaks. These tumors are not hypermutable and, thus, are less likely susceptible to immune checkpoint therapy. They remain sensitive to 5-fluorouracil, but patients show poor survival with advanced disease and higher levels of metastases. EMAST was found more frequently in colorectal cancers in patients of African descent in the United States.

Microbiome Environment and Colorectal Cancer

CRCs develop initially from precursor adenomatous polyps, may then progress in size, acquire additional genetic alterations ultimately resulting in carcinoma formation, and continue to evolve genetically to metastasize (1). At any step from normal colonocyte to adenoma to carcinoma to metastasis, the environment may alter conditions that either favor or reduce progression (Figure 4). For CRC, the local microenvironment consisting of microbial and inflammatory components has been shown and further hypothesized to influence the formation of CRC. Indeed, microbial components have been physically associated with each histological stage of CRC progression, from normal epithelium to

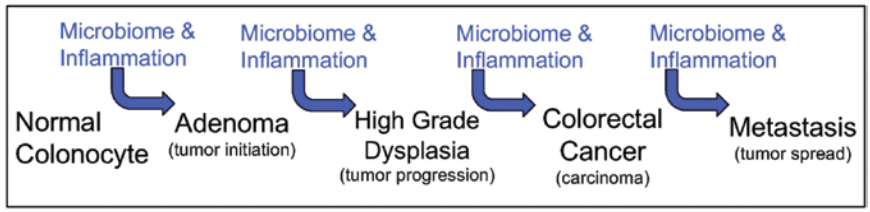

FIG. 4. Schematic of Microbiome Interaction During the Pathogenesis of Colorectal Cancer. The gut microbiome, shaped by diet and metabolism as well as potential genetic changes in the gut, can modify by promoting, accelerating, or potentially decelerating colorectal cancer at any and all stages of progression including metastasis.

adenoma to carcinoma (42). In particular, at the early steps of colonic neoplasia, pks+ *Escherichia coli* producing the toxin colibactin and enterotoxigenic *Bacteroides fragilis* (*ETBF*) producing the Bft toxin show evidence of microbial enrichment and linkage epidemiology; likewise, *Fusobacterium nucleatum* producing the effectors FadA and Fap2 has been associated with later steps of colonic neoplasia progression (42). Dejea et al showed a higher presence of both pks+ *E. coli* and *ETBF* biofilms in the colons of familial adenomatous polyposis coli patients over controls (43). Using *APC* mutant mice, intestinal infection with pks+ *E. coli* alone and *ETBF* alone allowed mice to survive ~110 days, but infection with both pks+ *E. coli* and *ETBF* shortened mouse survival to 82 days (43). Likewise, combined infection of mutant *APC* mice with pks+ *E. coli* and *ETBF* synergistically increased colon inflammation over infection with either bacterium alone (43). Using IL17-null mice compared to wild-type mice, infection with both pks+ *E. coli* and *ETBF* failed to generate tumors, indicating that cytokine generation and inflammation are critical for tumor growth (43). Overall, the combination of pks+ *E. coli* and *ETBF* increased colonic inflammation, generated faster tumor onset, showed poor survival, and was IL17-dependent (43).

Biofilms assessed from human sporadic CRCs showed that *F. nucleatum* was strongly associated with proximal (right-sided) CRCs (44). Indeed, the risk of proximal CRC was five-fold higher with a biofilm containing *F. nucleatum* (44). It is not clear how *F. nucleatum*, an oral anerobic bacterium, gets to the colon, but certainly the right side of the colon is more anerobic for it to survive and exert influence on biology. The biofilms containing *F. nucleatum* triggered immune responses, including IL6 release and downstream STAT3 activation, as well as increased normal colonic proliferation (44). Another study retroactively assessed an empiric dietary inflammatory pattern (EDIP) score, based

on the weighted intake of 18 foods constructed to predict plasma level increases of pro-inflammatory cytokines such as IL6, among patients with CRC (45). An association of *F. nucleatum* with the EDIP score was shown in patients only with proximal CRCs, but not from distal or rectal sites (45,46). Supernatant from *F. nucleatum*-infected permissive cells triggers DNA double strand breaks, indicating the potential for *F. nucleatum* to cause DNA damage and subsequent mutation (47). In assessing the association of *F. nucleatum* infection and genomic instability, *F. nucleatum* is most associated with MSI-H CRCs followed by EMAST CRCs (Table 4) (47,48). Additional genetic correlations demonstrate a strong association of *F. nucleatum* infection with the sessile serrated pathway for CRC pathogenesis occurring principally in the right colon (Table 4) (48). Sessile serrated pathogenesis includes hypermethylation of *MLH1* (causing MSI-H) and *BRAF* mutation (Table 4) (48). A weaker association with *KRAS* mutation suggests that *F. nucleatum* infection of these tumors is by a different mechanism, perhaps less causative, than the association with MSI-H, methylated *MLH1*, and mutation of *BRAF* (Table 4) (48). *F. nucleatum* infection among precursor adenomas demonstrated much lower infection loads compared to CRCs and no significant association, suggesting *F. nucleatum* infection might not be established at the adenomatous stage (48). *F. nucleatum*

TABLE 4

Univariate Odds Ratios of Colorectal Cancer-Associated Genetic Pathways and Colon Location with Fusobacterium Nucleatum Infection Among 304 Sporadic Colorectal Cancers With 36% Overall Infection Rate

Genetic Pathway or Site Status	Odds Ratio for *F. nucleatum* infection
MSI-H versus MSS	4.34
L/E versus MSS	1.90
MSI-H versus L/E	2.28
MLH1 hypermethylation versus not	2.84
$BRAF^{V300E}$ versus WT	2.39
$KRAS^{mut}$ versus WT	1.74
Colon versus rectum	2.50

The data demonstrate a strong association of *F. nucleatum* infection with the sessile serrated pathway of colorectal cancer pathogenesis occurring principally in the right colon. Multivariate analysis demonstrated that MSI-H, $BRAF^{V300E}$, *MLH1* hypermethylation, and $KRAS^{mut}$ are independent factors for *F. nucleatum* infection. Data obtained from Reference 48.

Abbreviations: MSI-H, microsatellite instability-high; MSS, microsatellite stable; L/E, microsatellite stable-low/EMAST.

infection can be shown in primary CRCs and in paired metastasis (49). In an attempt to understand if *F. nucleatum* recolonized the metastasis or traveled with the metastasis, patient-derived xenografts that were infected with *F. nucleatum* were serial passaged through eight generations of mice and evaluated for *F. nucleatum* presence by PCR and culture. Serial passage suggests that *F. nucleatum* traveled with the metastasis, as *F. nucleatum* could be cultured through passage #4 and *F. nucleatum* PCR could be detected through passage #8 (49). As *F. nucleatum* is an oral anerobic bacterium, treatment of mice containing the *F. nucleatum*-infected, patient-derived xenografts with metronidazole reduced tumor volume by 30% by day 23 of xenograft establishment over untreated mice (49). This finding suggests that *F. nucleatum* is a contributing factor for tumor growth in synergy with the malignant epithelium. In total, *F. nucleatum* causes DNA damage and appears to accelerate growth and metastases of human CRCs.

SUMMARY

The macro- and microenvironment play a key role in CRC formation, progression, and metastases. Understanding the roles the environment plays and the pathways involved will afford intervention strategies, including diet intervention, use of aspirin, and nonsteroidal anti-inflammatory agents, microbiome intervention, and prevention or modification of inflammation.

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DISCUSSION

Moore, New York: Thank you for your beautiful report. I'm a breast cancer oncologist. We speak a lot about hormone replacement therapy with our patients. I think I saw two different associations in your slides: one where hormone replacement therapy decreases risk of colon cancer and another where it increases risk of colon cancer.

Carethers, San Diego: No, they both decrease.

Moore, New York: Hormone replacement decreases risk of colon cancer? That's good news for our patients.

Carethers, San Diego: I don't know if every oncologist advocates that, but there is epidemiologic evidence that it does, so the premenopausal woman has a slower risk for colon cancer and hormone replacement therapy. We basically replicated this by epidemiologic studies.

Moore, New York: Thank you.

Blumenthal, Baltimore: Hi, John. That was a great talk. I was wondering if you could help me as a cardiologist try to make sense of the aspirin data. When we established our primary prevention guidelines for cardiovascular disease, we relied on three mega trials in 2018, one of which was the ASPRE-trial that actually showed an increase in cancer deaths with aspirin. Two other trials looked at more middle-aged individuals. How do you feel now about aspirin? Do you think its effects may differ based on a person's age and cancer risk?

Carethers, San Diego: I'll just give you my cliff notes on this. You are right about the ASPRE-trials. Aspirin epidemiologically has been associated with low risk of cancer, but the biologic endpoints have been short. In papers that were published 15 or 20 years ago in the *New England Journal of Medicine,* the endpoints were five years or seven years when it takes much longer for cancer to develop. Regarding the ASPRE-trial, if you give aspirin to elderly patients, the number one cause of death after aspirin was not bleeding, it was cancer. If you look at everything in totality, there's probably a sweet age spot of taking aspirin. I personally started taking it around age 51, and it takes about 10 years to see the effect on reducing cancer risk based on Andy Chan's data. Once you get past a certain age, the effect likely drops, and there's probably some escape from the protection. The sweet spot is probably somewhere between the ages of 50 and 65 or 66. Based on the available data, if you take it at 65, you will likely have the effect going to 75 or 80. That's kind of my plan right now too.

Limacher, Gainesville: I think it gets complicated. Thanks so much for a very comprehensive and enlightening presentation. I also want to come back to the prevention recommendations. How do you address the findings from the Women's Health Initiative Clinical Trials which had negative outcomes for hormone therapy and for calcium plus vitamin D on cancer outcomes?

Carethers, San Diego: That's a good question, and I don't have a full answer to that. I will say that epidemiologically it's been positive, but when you look at some of these

outcomes you need to focus on the details—just like we were talking about with aspirin. Again, I don't know if anyone out there is saying go on hormone replacement therapy or don't go on hormone replacement therapy solely for colon cancer prevention. I would not necessarily advocate for that. We need better studies on actual longitudinal data. Clarification is starting to come out, not just with epidemiologic studies, but with actual trial studies that are showing some of these differences.

Wilson, Durham: That was a beautiful and brilliant talk and thank you very much. I wanted to make a comment and ask a question. When the new wave of studies of the microbiota burst forth in the 1990s, a lot of the old knowledge base was lost. For instance, it has been known since the 1960s how to cultivate most of these organisms, but the new group of people just didn't seem to understand that. In the 1980s, Tracy Wilkins at the Virginia Bioinformatics Institute found that if you fed people a high fat diet they developed a powerful mutagen in their stool that could be detected and he traced it down to a eubacterium. I think it was *Eubacterium lentum.* Has this ever gotten into the more recent literature, and is anybody looking at it?

Carethers, San Diego: Not that specific one that I know of.

Post, Baltimore: Thank you for a really nice talk. Could you speak briefly about the risk for colon cancer in patients with ulcerative colitis now that many patients are treated effectively with biologic therapies that dramatically decrease inflammation, and how has that changed the epidemiology?

Carethers, San Diego: Ulcerative colitis, as many of you know, is an autoimmune disease that can cause repeats bouts of bleeding, but the chronic inflammation raise one's risk for colon cancer. In Crohn's disease, particularly in Crohn's colitis, the level of treatment now used to reduce inflammation has reduced the risk of cancer. In fact, we've done studies in which we looked at some of these frame shift microsatellite markers and showed that for people who are taking medications like mesalamine and other things to reduce inflammation the rates of those mutations go way down. Chronic bouts of inflammation are what helps to continue to drive the risk for cancer; as long as you're controlling the inflammation, your risk of cancer should go down. In fact, that seems to be proven as data come out with these newer biologics that people are on for much longer periods of time. We published a study regarding ulcerative colitis using EMAST microsatellite markers and showed that if you have ulcerative colitis without any dysplasia, or if you have someone who has dysplasia, they have more of these frame shift mutations. If you have someone with cancer, they have even more. It's almost like a biological marker for progression, even though those frame shift mutations aren't necessarily what's driving the dysplasia-associated cancer. We also looked at this in short- and long-term ulcerative colitis patients. Clearly, long-term patients have a lot more of these mutations. The longer you have it, particularly if it's not as controlled, you're more likely to get more of these mutations and change your risk. As long as you're keeping the inflammation under control, your risk is dropped and epidemiology data show that. So now we're seeing fewer of these cancers over time.

Zeidel, Boston: John, terrific talk. You've taken a very complicated topic, especially for someone like me, and made it straightforward, or at least understandable. Very quick technical questions. The fusobacteria studies are very interesting. When *E.coli* infects your urothelium, it enters the cell and forms a little colony inside the cells. Is it possible that the fusobacteria are actually in the epithelial cell, these cells metastasize and then carry the fusobacteria with them, and the fusobacteria are coming out of the cell to reinfect and that's where the metronidazole is doing what it's doing?

Carethers, San Diego: Very possible. In fact, I always thought fusobacteria was more of an adherent, but it's actually minimally, minimally invasive. It can attach and a little bit get in, and this is how we are able to take all these fusobacterium copies from these colon tumors. You might think by processing that you wipe out all the bacteria, but they're actually minimally invasive. I think you're absolutely right in that possibility. We can detect it by PCR. We can detect how much is there. In a fresh specimen, there might even be more on the surface, but it gets wiped away. The Cynthia Sears Lab studied the biofilm and showed it was on the surface. Some of the studies I referenced are from formalin-fixed specimens and picked the fusobacterium that had minimally invaded the epithelium.