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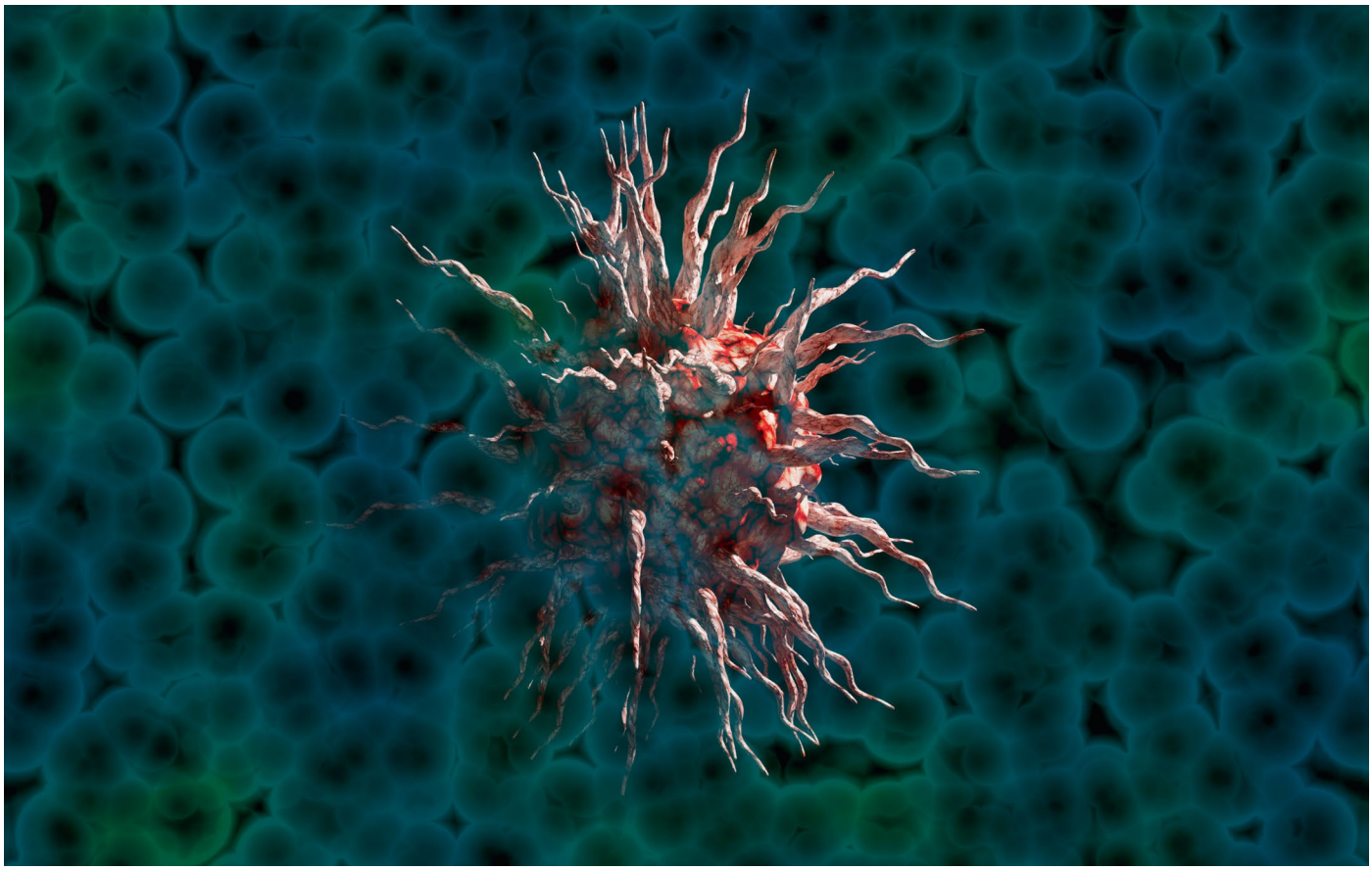

CANCER DEBUGGED

Tumors are rife with bacteria and fungi. Their ubiquity is proving useful in detecting cancers, categorizing them, and even determining whether certain interventions will work.

By Monique Brouillette

Last July the first predictive clinical test based on the gut microbiome was approved in Europe. The test, called BiomeOne, was developed by Vienna-based biotech BiomeDx. It analyzes the DNA in gut microbes of patients with cancer and predicts who are most likely to have success with immunotherapy. The test has a prediction accuracy of over 85% and offers hope that it will guide cancer treatment and expand the population for whom immunotherapy is helpful.

BiomeDx joins a handful of other microbiome-based analytic biotechs that aim to offer predictive and diagnostic tests for cancer. Pending a series B funding round, Metabiomics hopes to launch a clinical trial on its non-invasive colon cancer test, which earned breakthrough device designation from the US Food and Drug Administration (FDA) in 2019. And last August another US company, Viome, launched a test, CancerDetect, that analyzes the microbial community transcriptome (metatranscriptome) of saliva to detect oral and throat cancer. This new generation of microbiome-inspired clinical tools promises to be non-invasive, relying only on samples such as stool, blood or saliva. And biotechs are racing to take advantage of these tools. "I think that it's certainly worth doing," says Steven Salzberg, computational biologist at Johns Hopkins. "But you have to be really skeptical of what you find." Before they become widely adopted, the field needs validation, replication and large gold standard trials.

A drop of prevention

Gregory Sepich-Poore was awe-struck when he first heard about a puzzling link between microbes and cancer. In 2017 the MD PhD student was doing graduate work at the University of California, San Diego, when cancer biologist Ravid Straussman at the Weizmann Institute showed that human pancreatic ductal adenocarcinomas are loaded with bacteria. Not only that, among them are species that produce enzymes that degrade the commonly prescribed cancer drug gemcitabine. The link

Table 1 | Programs analyzing tumor microbiota

Company	Product/stage	Focus	Total funding
BiomeDx	BiomeOne/Approved in European Union with CE-IVD designation	Response to immunotherapy for three cancer types	Austrian Research Promotion Agency
Metabiomics	LifeKit Prevent/In clinical trials	Microbial biomarkers in stool samples to identify colorectal polyps	$30 million
Micronoma	Oncobiota/Breakthrough designation	Detection of early-stage cancer by liquid biopsy, focusing on lung cancer	$17.5 million
Viome	CancerDetect/Breakthrough designation	Early-stage oral and throat cancer	$150 million

was one of the first to suggest that microbes in cancer might affect efficacy of treatment.

Years before, Sepich-Poore's grandmother had succumbed to pancreatic cancer after she became resistant to the treatment. This research provided a possible reason why her treatment had failed, and the young biologist vowed to spend his years in graduate school understanding the connection. He joined the lab of Rob Knight and scoured a database of 20,000 cancer samples from The Cancer Genome Atlas, looking for evidence of bacteria. According to Sepich-Poore, the project was the "computational equivalent of trying to find needles in a haystack where there are more straws of hay than stars in the Milky Way."

He was shocked to find as much as 2% of the six trillion reads in the atlas were bacterial, and 33 types of cancer contained microbial DNA or RNA. Each distinct cancer had a unique combination of microbes. Even tumors that arise in the same tissue were distinguishable: lung adenocarcinoma and lung squamous cell carcinoma had distinct signatures. More intriguing from a diagnostic standpoint was that the tumor microbial fragments were detectable in the blood, offering the exciting prospect of a non-invasive blood assay. The group was also able to verify well-known associations between other human pathogens and cancer: they found cervical and thoracic cancers enriched in human papillomavirus and liver cancers associated with hepatitis viruses. They published the findings in *Nature* in March 2020.

"It was remarkably surprising," Sepich-Poore said. The research showed that no cancer type was truly sterile. It also offered tantalizing hopes of finding better ways to detect the disease. "This suggested that there might be an opportunity to distinguish between cancers because different tumor types have different microbiomes associated [with them]". Several months later, he and Knight set out to design a diagnostic and launched Micronoma (Table 1).

"It's impressive," says Georg Zeller, computational biologist at the European Molecular Biology Laboratory. "This is a really robust signal they found here," he says.

The results generated a lot of excitement, but also raised skepticism. In a comment published in *Cancer Cell*, Salzberg and his Johns Hopkins colleague Cindy Sears wrote that the work offered a "strong foundation on which to begin," but was "not prime time yet."

"I understand that companies are excited and they're trying to find ways to use this powerful sequencing technology," says Salzberg. "But I think it's really hard work to come up with a genuine correlation between bacteria or viruses in any cancer." A major challenge is contamination, according to Salzberg. Bacteria cover every surface in the laboratory. There is no readily available way to completely remove them from bottles, dishes, reagents and samples. One can sterilize and kill bacteria so they are no longer infectious, but that does not remove the bits and pieces of bacterial DNA throughout the various stages of the experiment. In the paper, for example, they found so much bacteria in the control samples that they had to discard as much as 90 percent of microbial data due to concerns that they represented contaminants.

"It's very hard to know if the signature you've got is a significant one, even if you do careful, standard statistical tests," Salzburg says. He is developing his own metagenomic test to detect infectious disease, and he treads carefully and with a lot of skepticism, even in his own work. He focuses on detecting infectious agents in situations where microbes are expected to be in high concentration, which is easier than looking for small signals, like the ones generated by sequencing tumors. Looking at complicated mixtures diminishes signal strength, whereas looking for a single microbe with a strong signal in a tumor, like human papillomavirus in cervical cancer, is more reliable, he points out.

Despite the apprehension, studies keep coming up with more evidence that tumors are harboring their own microbial communities. Just a few months after Knight's paper, Straussman's group published similar results. The group obtained tissue from 1,500 tumor samples and used immunohistochemical techniques to show that the bacteria are located inside tumor cells. They created a catalog with seven types of cancer and confirmed that tumor types had specific microbial signatures. The research was validated by hundreds of controls to rule out contamination; they even tested the paraffin wax blocks in which tumor tissues were embedded.

A year later, they published a study of human melanoma metastases that hinted at a potential mechanistic link between the presence of tumor microbiota and disease progression. They showed that hundreds of peptides derived from intratumoral microbes are present on the surface of cancer cells, as well as on antigen-presenting cells (Fig. 1). This raised the possibility that the peptides can both elicit immune responses by being presented on antigen-presenting cells and be a target of the immune system by being resident on tumors. According to Sepich-Poore, this research went one step further toward showing how intratumoral bacteria can affect response to immunotherapy by engaging the immune system. "It's the best mechanism that I am aware of today," he said.

Gut check

Although a steady stream of research shows a link between the microbiome and tumors, some warn that it is still early days. Curtis Huttenhower, a computational biologist at the Harvard T.H. Chan School of Public Health, is one. "Part of what makes that exciting is the potential … it's a cool idea," he says. "It's much shakier than something more robust, like stool microbial sequencing."

Huttenhower focuses his research on the gut microbiome and its role in causing colorectal cancer. He says the gut microbiome offers many advantages over the tumor microbiome. For one, the research is far more established. In the ten years since researchers began cataloging gut microbes, they have been able

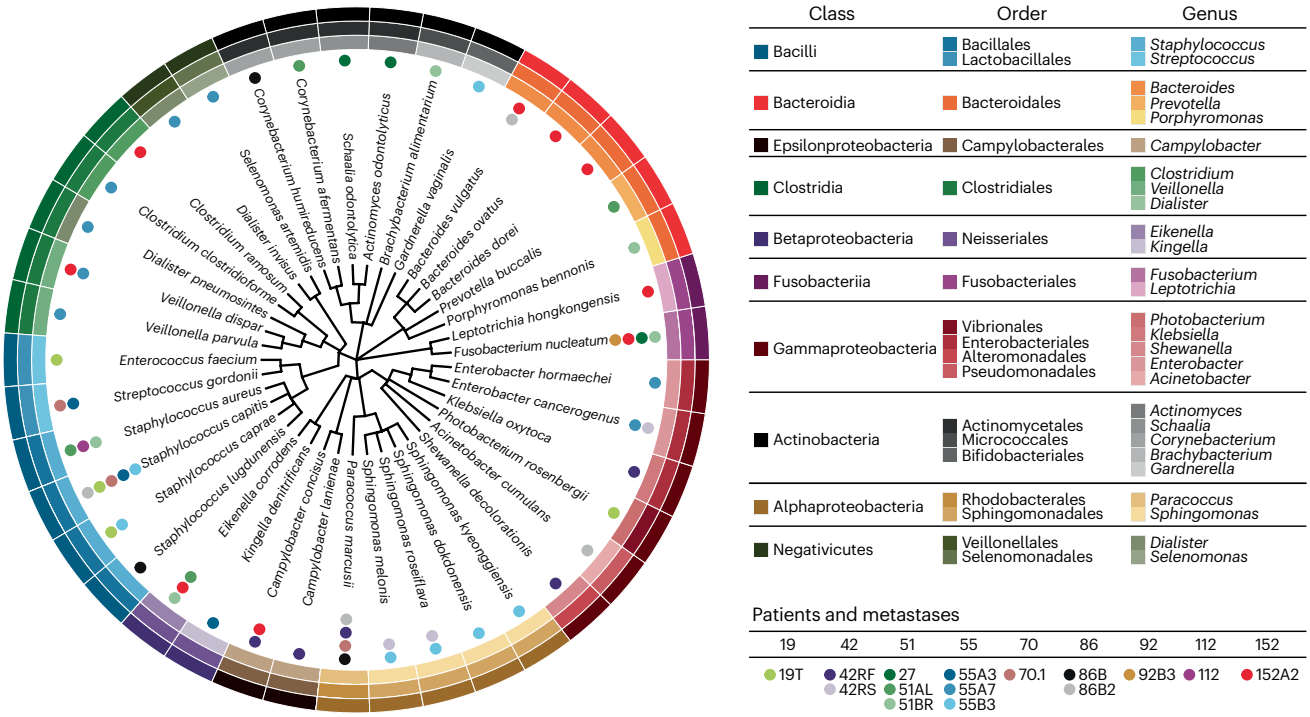

Fig. 1 | Intratumor bacteria in melanoma. Phylogenetic tree of the bacteria found in 17 melanoma metastases originating from 9 patients. The analysis is based on ribosomal 16S RNA gene sequencing. Reproduced with permission from S. Kalaora et al. *Nature* **592**, 138–143 (2021).

to find links of specific microbes to cancer. In addition, Huttenhower says the tools for studying the gut microbiome have been honed and refined over the past decade.

What has emerged are links between whole microbial communities and cancer, changes in community composition over time, and even associations between individual species and cancer. Certain species of sulfide-producing bacteria, for example, are associated with a higher risk of colon cancer because they produce inflammation-inducing sulfide. Also, *Fusobacterium nucleatum*, a bacterium typically found in the mouth, creates an inflammatory environment known to aid in tumor growth. To date, scientists have homed in on a dozen or so bacteria that may promote tumor growth and formation.

Another advantage of studying gut bacteria is the sheer volume and density of microbes that reside there. The concentration of gut bacteria is greater than those of all other microbiomes in the body, including the mouth and the blood. "It's like looking at the difference between a dim star and the moon," says Huttenhower. "One of them is easy to do and the other is difficult."

Because of these advantages, gut microbiome-based tests are leading the way. BiomeDx developed their prognostic tool, Biome One, with a combination of bioinformatics and artificial intelligence that they trained on a dataset of stool microbiomes from 10,000 patients. The test, which looks at 16S ribosomal RNA sequences from over 1,400 species, has a sensitivity of over 85% and is validated for people with three types of late-stage cancers: non-small-cell lung cancer, renal cell carcinoma and malignant melanoma. Using machine learning, the test creates a microbial signature that shows the probability of having a response to checkpoint inhibition-based cancer immunotherapy like that directed at CTLA-4, PD-1 or PD-L1. In addition, they show the probability of immune-related adverse events. A patient would learn, for example, they have a 66% chance of response to PD-L1 and a 100% chance of adverse immune events. Information is also provided on the person's gut bacteria richness, diversity and even enterotype, which is a classification system for grouping intestinal microbiome compositions.

In addition to prognostics, companies are also developing non-invasive diagnostics. Metabiomics' colon cancer screening test, called LifeKit, uses a bioinformatics approach and analyzes stool samples for microbial biomarkers linked to colon polyps (precancerous adenomas) and carcinomas. If it successfully passes through clinical trials and FDA approval, the test will enable doctors to distinguish between polyps and cancer non-invasively. The current non-invasive tests on the market include fecal immunochemical tests and Cologuard, which detect blood in the colon and human genetic mutations associated with colon cancer. They are good at finding late-stage cancers. Metabiomics hopes their test will fill the gap, allowing people at high risk to test more frequently than the recommended schedule for colonoscopies, obtaining a result that distinguishes between polyps and cancer. At the same time, Kuehn says they want their test to "broaden the screening program" by providing better sensitivity and specificity. But that path to FDA approval can be long and challenging.

The company plans on enrolling 3,000 participants around the globe in a large validation study. Called Collect, it will enroll people at high risk previously diagnosed with colon cancer or precancerous polyps, along with those at average risk, and will help train the algorithm. When that is complete, the company is planning a larger clinical trial, called Prevent, that will enroll 12,000 participants aged 40 years or older who are scheduled for a regular colonoscopy. The trial is meant to validate the test for clinical use in a general population.

Statistically speaking

Metabiomics is leading the pack in seeking regulatory approval by planning large clinical trials. The most difficult task, according to Levi Waldron, an epidemiologist at the City University of New York Graduate School of Public Health and Health Policy, is validating the test in large populations of healthy people as well as those with cancer. He says that algorithms commonly fail in the transition between small training studies and their deployment in the general population. The small training populations are typically enriched in people with cancer and doesn't accurately reflect the general population, in which cancer incidence is quite low.

"We've seen problems with it before like when screening for breast cancer or prostate cancer – that if you start screening in a healthy population with a low incidence of cancer, you get a lot of false positives," Waldron says.

Others are trying to increase the general knowledge of the microbiome in hopes it will push the clinical applications along. Huttenhower thinks the field will benefit from large prospective studies that link diet, lifestyle, microbiome and disease. He is leading a trial now called Microbiome Among Nurses (Micro-N). It will collect microbiome data on 20,000 participants from the Nurses' Health Study II, one of the largest and longest-running population-based studies of chronic disease in women.

The Nurses' Health Study II, launched in 1989, gathers lifestyle and health data through questionnaires and biospecimen samples like blood and urine. Micro-N, launched in 2019, will combine historical data from the Nurses' Health Study II with microbiome data from donated stool and saliva in a subset of participants. So far, they have enrolled 18,000 participants and plan to follow them for the duration of their lives. The study will measure changes in their microbiomes as they age and acquire disease.

Companies developing these tests could take a lesson from the FDA-approved non-invasive Cologuard test, which went through rigorous validation studies before gaining FDA approval in 2014. A team led by David Ahlquist, a former Mayo Clinic gastroenterologist, and the company Exact Biosciences recruited 10,000 participants, 65% of whom had colorectal cancer and 8% advanced precancerous lesions. They designed controls, collecting fecal samples in a manner consistent with sample collection in clinical practice. The test, which combined molecular assays for cancer mutations and methylation patterns, was compared to a fecal immunochemical test. The study found that the Cologuard test had a sensitivity of 92%, whereas the fecal immunochemical test had a sensitivity of only 74%. Zeller says this study, published in the *New England Journal of Medicine* in 2014, is the "gold standard." He thinks microbiome-based diagnostics will need to undergo similarly rigorous testing to gain FDA approval.

It takes multitudes

As scientists continue to probe the hidden world of our microbiome with better tools, they are finding that the bacterial communities aren't the only inter-species interlopers that impact cancer. Sepich-Poore and collaborators from the Weizmann Institute of Science, University of California San Diego and Micronoma screened 17,000 tissue and blood samples from four cancer cohorts looking for evidence of a mycobiome, or fungal biome. Using a combination of open-ended and targeted sequencing techniques, the latter using fungal primers, they found fungi everywhere. Although present in lower abundance than bacteria, fungi were present in all 35 cancer types they screened. What was most surprising, according to Sepich-Poore, was that even though there was less fungal genetic material than bacterial, its source organism was informative in determining tumor type. They published their results in the journal *Cell*.

"When we combined the fungal information with bacterial information in the same samples, it actually led to synergistic diagnostic performance improvements," Sepich-Poore said. Thus, not ten years after he first learned of the presence of gemcitabine-eating microbes in pancreatic cancer cells, he found microbes in all types of cancer hiding in plain sight in large genome databases.

And as this field grows, so too do the variety of clinical tools in development. In August, the startup Viome launched a saliva-based test that claims to detect oral and pharyngeal cancer by analyzing microbial and human RNA. The screening test, built on the company's mRNA technology and artificial intelligence platform, is not FDA approved, but is marketed as a laboratory-developed test under an FDA Breakthrough Device designation gained in 2021. Oral cancer is difficult to detect in its early stages, and the test may offer a way to non-invasively screen before symptoms arise. It has a sensitivity of 83% for early-stage cancers and 92% for stage 1 cancers, with a specificity up to 98%. According to CEO Guru Banavar, the technology is based on data from half a million samples from people in over 100 countries. Banavar says what distinguishes this test from others in the field is that it takes a top-down approach that uses machine learning to characterize gene expression patterns rather than biomarker or community composition.

Waldron says that the test's sensitivity is far too low to use for screening of the general population and would result in many false positives. For him, it is unclear how it would be used, aside from maybe an intermediate step in people suspected of cancer before they undergo invasive biopsy. However, he cautions that he can't imagine anyone undergoing cancer treatment without a gold standard biopsy. "It's fine to be excited for this test, but it going to be years down the road, and it still has to prove itself," he says.

After two decades of intense study, knowledge of the microbiome may be finally enabling clinically useful tools. Thanks to next-generation sequencing techniques like metagenomics and computational tools like artificial intelligence, scientists are finding microbial signatures that can inform cancer diagnosis and even response to therapy. The approaches are still unproven, but they may offer a new arsenal from which to fight back against cancer. First, though, the tests must be validated.

"No single solution will fix everything about cancer," said Huttenhower. "A little bit of everything that works is going to be good eventually … we just have to make sure it works."

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