DDN

EXPLORING DRUG DISCOVERY AND DEVELOPMENT

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A DEADLY RISE

SYMPTOMATIC RABIES HAS NO CURE, BUT RECENT ADVANCES IN RABIES STRUCTURAL BIOLOGY AND ANTIBODY COCKTAILS POINT TO BETTER TREATMENTS ON THE HORIZON.

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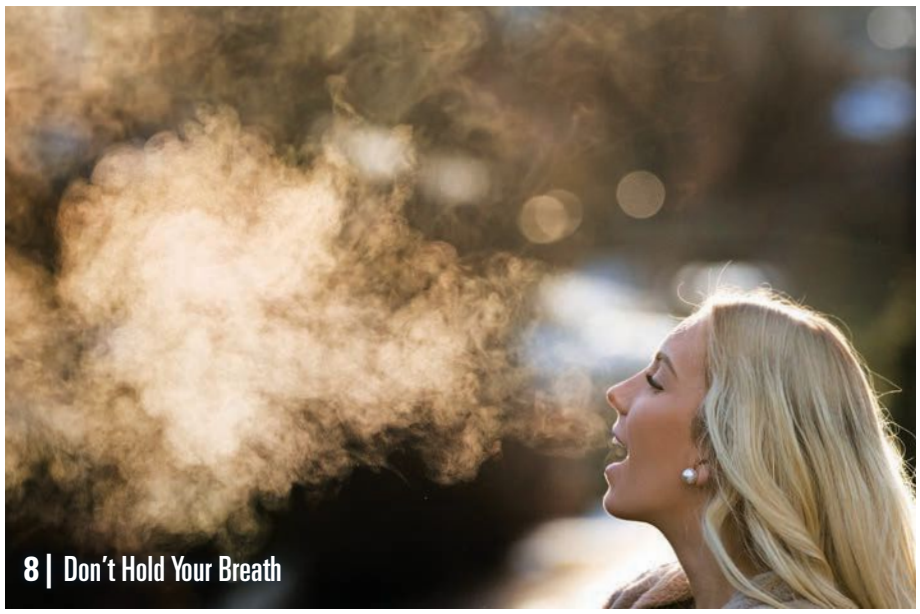

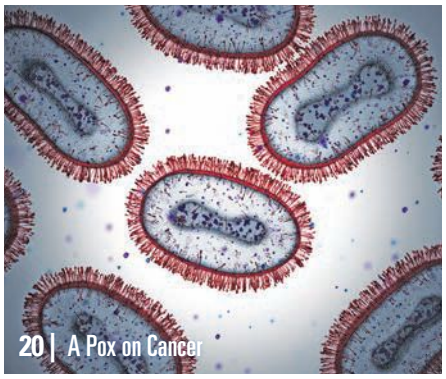

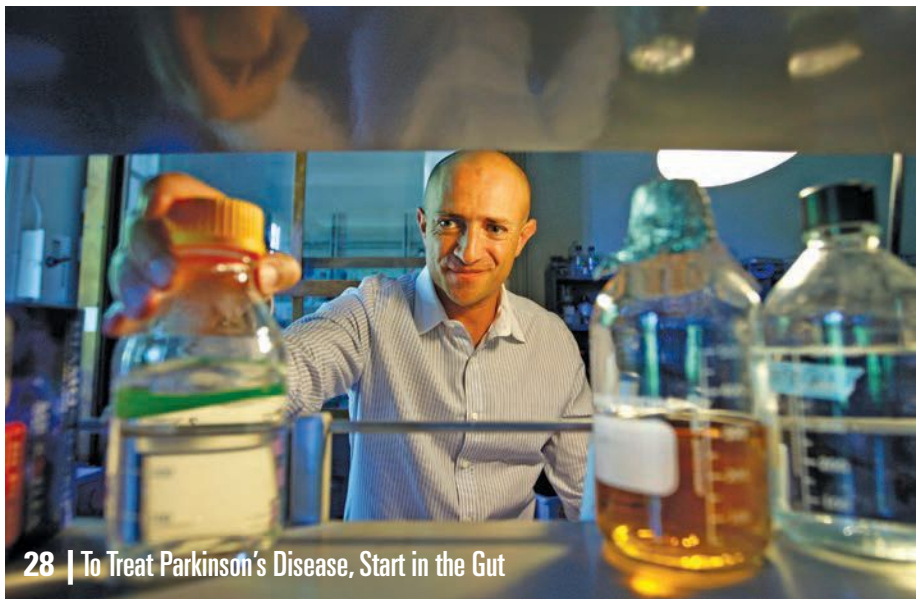

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Conferences and COVID-19

Stephanie DeMarco, PhD
ASSOCIATE EDITOR

As I pushed open the glass doors of the conference hall, I felt the familiar rush of excitement mixed with nerves unique to scientific conferences. People milled around the lobby, chatting with one another, poster tubes slung over their shoulders. Others hustled toward the escalators, laptops tucked under their arms. I slung my press pass over my neck and joined them, ready to soak up a week of exciting new science. But COVID-19 was never far from my mind.

While many people in my local community have stopped wearing masks inside, I've been slower to let my guard down. I have a close family member who is immunocompromised, and my family and I have spent these past three years of the pandemic being very careful to not get him sick.

Knowing that this conference would be the biggest COVID-19 risk I'd taken all pandemic, I did my best to prepare: I got the new bivalent COVID-19 booster and flu shot a couple of weeks before the meeting and restocked my stash of heavy duty KN95 masks.

Just as I'd hoped, the conference filled my cup with exciting new findings and experimental techniques that I can't wait to share in *DDN* in the coming months. But the general lack of masking within the packed seminar rooms and poster hall surprised me. From my rough estimate, about a quarter of the attendees wore a face covering of some sort inside.

About three days after I returned home from the conference, my cell phone buzzed. An unfamiliar pink icon and a long block of text lit up my screen: *California Department of Public Health - Possible exposure to COVID-19 virus.* My heart sank.

As each rapid test and PCR test turned up negative, it seemed like I was spared. But a quick perusal of the conference hashtag on Twitter told me that other attendees were not so lucky.

Nothing can replace the organic interactions and joy of seeing old colleagues at in person scientific meetings. Some of the most fruitful collaborations and epiphanies happen at poster sessions or over coffee. But the risk of COVID-19 is still very real. Viruses don't care about new conference friendships. They just want a new host to infect.

For many researchers who are immunocompromised or have family members who are, in person meetings are still inaccessible and will remain so for the foreseeable future. Even for healthy people, a mild COVID-19 infection can lead to long COVID and other long-term effects that scientists are still trying to understand.

There are many science backed precautions that conference organizers can impose to reduce the risk of COVID-19 among attendees. Requiring COVID-19 vaccination — which prevents severe disease but not necessarily infection — is a good start, but an indoor masking requirement is even better. Asking attendees to take a rapid antigen test before traveling to a conference is one simple precaution and having free or low cost testing available on site is another.

We will likely be living with COVID-19 for a long time, so how will we, as members of the scientific community, respond to it — with a shrug or with a plan?

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tools & techniques

Stomach Bugs

New bioengineered bacteria can sense and report on conditions in the gut.

BY HANNAH THOMASY, PHD

IN 2009, ARTISTS ALEXANDRA Daisy Ginsberg and James King teamed up with synthetic biology students at the University of Cambridge to create the Scatalogue. The students engineered *E. coli* to respond to a stimulus by changing color, creating a signal robust enough to be seen with the naked eye. The team envisioned a future in which these bacteria could detect problems in the gut, turning different colors to indicate different diseases; the artists brought the concept to life in the Scatalogue art project, a collection of cheerily colored (fake) feces to represent this hoped for advance in diagnostics.

While the Scatalogue isn't yet a reality, it might not be far off. Advances in synthetic biology and expanded understanding of the gut microbiome enable researchers to create increasingly more sophisticated bacterial sensors that can recognize and record conditions in the complex environment of the human gut. While fecal analysis gives researchers a good idea of what's happening at the far end of the gastrointestinal tract, it doesn't necessarily indicate what's happening in the several other feet of intestines between mouth and rectum (1). Scientists hope that one day their genetically engineered bacterial systems will enable early diagnosis and continuous monitoring for gut conditions such as inflammatory bowel disease and cancer.

Florian Schmidt endows cells with the ability to store "memories" of gene expression in Randall Platt's lab at the Swiss Federal Institute of Technology.

CREDIT: BOTNAR RESEARCH CENTRE FOR CHILD HEALTH

The first steps

Harvard University biologist Pamela Silver spent the first part of her career studying the movements of proteins and RNAs within cells; her early work formed the basis for a drug that is currently approved to treat some blood cancers. In the early 2000s, she was ready for a change. She linked up with the Synthetic Biology Working Group at the Massachusetts Institute of Technology (MIT) and decided to pivot her team to work in a new field.

She started thinking about engineering bacteria to sense specific things in their environments. Applying these biosensors in the gut, she said, "seemed kind of obvious. The gut is a key environment where bacteria live, including *E. coli*. One of the tenets of synthetic biology is that we know a whole lot about engineering certain organisms, and one of them is *E. coli*."

Bacteria evolved many ways to sense their environments; in one well characterized system, a specific promoter drives expression of a downstream gene in response to a drug called anhydrotetracycline. Silver decided to use this system in the first proof of concept study to create a bacterium able to sense conditions in the gut.

> "The beauty of the lambda switch is that once it's on, it stably stays on."
>
> – Pamela Silver, Harvard University

She didn't only need the bacteria to respond to chemicals in the gut; she also needed them to "remember" the information. To endow the bacteria with memory, Silver borrowed a genetic switch from another model organism: bacteriophage lambda. The switch consists of two elements, cI and cro. Expression of one of these genes strongly represses transcription of the other, so the system is stable in the cI state ("off") and in the cro state ("on"). "The beauty of the lambda switch is that once it's on, it stably stays on," said Silver.

Silver engineered the bacteria so that the promoter would drive expression of cro, flipping the lamba switch to the cro, or "on," state. She also added in a cro-driven reporter gene that would tell them when the switch had been flipped on.

Then came the test. Silver gave the engineered bacteria, all of which began in the "off" or cI state, to two groups of mice. One of the groups also received a low dose of anhydrotetracycline. When the researchers checked the bacteria coming out the other end, they found that they stayed in the "off" state in mice that hadn't received the drug. However, in the other group of mice, the engineered bacteria reliably sensed the presence of the drug, flipping the switch to the "on" state, demonstrating that these bacteria could sense, remember, and report on conditions in the gut (2).

Trust your gut

In the years since that landmark study in 2014, research on bacterial biosensors has blossomed. Researchers moved from proof of concept studies in which bacteria were triggered by a drug to engineering bacteria that could sense molecules relevant for human disease.

Scientists aren't engineering these sensors from scratch, though. For the most part, they're borrowing two-component systems that already exist in nature. Two-component systems, explained Jeffrey Tabor, a bioengineer at Rice University, "are the primary means that bacteria use to sense their environments. They're bacteria's eyes and ears for their world."

These systems generally consist of a sensor protein in the cell membrane, which activates in response to a specific stimulus. This protein transfers the signal to a second protein, which turns gene expression on or off, allowing the cell to respond appropriately to the stimulus.

Even so, finding a two-component system that responds to a stimulus that is relevant for human disease isn't necessarily straightforward. In fact, Tabor's discovery of a sensor for a molecule involved in gut inflammation happened almost by accident.

For this project, he said, "we were originally looking for bacterial sensors of environmental pollutants." They noticed that one of the marine bacteria they were studying had a novel sensor that likely controlled an enzyme for using thiosulfate, which some bacteria can "breathe" when oxygen is absent. They guessed, correctly it turned out, that the sensor allowed bacteria to sense thiosulfate in the environment.

This wasn't particularly useful for their

E. coli ***can be engineered to report on stimuli in their environments.***

research on pollutants, but Tabor knew that thiosulfate also associated with inflammation in the gut. This sensor might be useful for monitoring inflammatory conditions in the mammalian gut.

Tabor borrowed this sensor from the marine bacteria and engineered it into a strain of probiotic mammalian gut adapted *E. coli* known as Nissle 1917. He also added a green fluorescent protein reporter.

Sure enough, when researchers administered these engineered bacteria to mice with experimentally induced colitis, the bacteria's trip through this inflammatory environment made them glow green (3).

While this experiment was successful, the technique needs some finetuning before it's ready for human use. One of the big challenges is making the signal easier to see. If the diagnostic is administered as a sort of bacteria laden milkshake, Tabor said, the first problem is that, "99 percent of those bacteria will die in the stomach, give or take. The ones that don't die get spread out along the 28-foot tube that is your gastrointestinal tract." This can result in a weak signal once the bacteria reach the far end. In the mouse study, scientists had to use flow cytometry, a single cell analysis technique, to identify the signal from the bioengineered bacteria.

Patients, and probably many general practitioners, don't have access to flow cytometry, so the team is currently working on ways to make the signal more visible. Tabor and his team are developing a strategy to stick the bacteria together using hydrogels, which will hopefully help more of them survive their journey through the stomach and keep them together so that the signal is concentrated enough to be visible to the naked eye once the bacteria are excreted.

Tabor imagines a product like this one day serving as an early warning system. "Say you're a Crohn's disease patient and your doctor asks you to drink this bacterial solution every morning at your house. And if your toilet water turns blue, you are having an inflammatory flare, and you need to go see the doctor."

Human gut disorders are complex and involve many more factors than thiosulfate. "We are pretty sure we're going to need to find more sensors from human gut bacteria to reliably sense human inflammation," said Tabor. "What we're doing is getting clinical samples — fecal samples from healthy people and sick people — and we're screening them against lots and lots of two-component systems from human gut bacteria in order to discover clinically translatable human inflammation biosensors."

> "Two-component systems are the primary means that bacteria use to sense their environments. They're bacteria's eyes and ears for their world." *– Jeffrey Tabor, Rice University*

Cell biographies

Although bioengineered sensors that can detect one or even a few different biomarkers could be useful as diagnostics or early warning devices, they can't really provide scientists with a complete picture of everything that's happening in the incredibly complex gut environment.

Randall Platt, a bioengineer at the Swiss Federal Institute of Technology, thinks he may have a potential solution. Platt had always been interested in CRISPR and gene editing, but when he founded his own lab in Zürich, he wanted to explore CRISPR's recording capabilities.

In nature, CRISPR-Cas systems take genetic information from an attacker, like a bacteriophage, and integrate it into the bacterial genome to provide the bacterium with a sort of immune "memory" of the attacker. Platt and postdoctoral researcher Florian Schmidt wanted to see if they could modify this system to record other types of "memories."

In 2018, they successfully used this system to grab a snippet of the cell's own RNA, convert it back into DNA, and integrate it into the genome, preserving this piece of transcriptional history (4). While most of the cells grab only a single snippet, by using a population of cells, they created a more detailed record of gene transcription, providing information about how cells responded to different environments. Even though this isn't a direct recording of the environment, a lot can be inferred about the environment by measuring how the bacteria respond.

This project started as a theoretical exercise; Platt's team just wanted to see if they could do it. "Once we succeeded, we realized how powerful it could be. We can create biographies inside of cells in any environment," said Platt. "My interest is in human health and trying to help people and patients. And so, we wanted to see if we could use this to gain some really valuable clinical information about what's happening inside of the gastrointestinal tract."

The research team found that in monocolonized mice (mice colonized with just the reporter bacteria), the bacteria recorded different patterns of gene expression based on the diets the mice ate, or whether they had experimentally induced colitis (5).

Platt said that the diet analysis "is where the technology really shines, because *E. coli* have evolved to be experts in adapting to different carbon sources." By analyzing transcriptional memory, researchers got clues about what carbon sources the bacteria used, which wasn't necessarily constrained to what the mouse ate.

"We could tell which diets were good for the mice to be eating," said Platt. "If the bacteria didn't like the diet, in the sense that it wasn't rich in a range of carbon sources and it was only enriched in bad things like starch or fat, the *E. coli* would start to eat mucus carbon sources. So essentially, if you don't feed the *E. coli*, they feed off of

Conditions in the gut are important for our overall health, yet historically, they have been very difficult to assess.

> "Bacteria aren't good at communicating over long distances. So, we had to figure out how to combine these together with some other modality, and electronics made a lot of sense."
>
> *– Timothy Lu, MIT*

the mucus and deplete that barrier." The protective layer of mucus lining the gastrointestinal tract is crucial for maintaining a healthy gut, so if bacteria consume it, this could lead to problems.

In the near future, said Platt, "we see these bacteria as a great way to make nutritional assessments in individual people, to figure out which diets are good for individuals to eat in a therapeutic context in which patients need appropriate nutrition to manage their other health conditions like cancer or diabetes."

Further in the future, Platt hopes that these reporter bacteria will help unravel some of the mysteries surrounding irritable bowel syndrome (IBS), the many varied causes and mechanisms of which are poorly understood.

"IBS is more of an umbrella term for a whole bunch of different conditions related to things like intolerances or malabsorption.… Part of the difficulty in labeling these as proper disorders might be that there's just no way to measure what's actually going on in the intestine," said Platt. "We believe if we put our tool in, we can start to understand the mechanistic basis of what makes one patient different from another and then know how to take action in each case."

A perfect partnership

Tabor's and Platt's approaches have their own advantages and specific use cases, but both techniques require the bacteria to exit the body before they can report on what they found inside. Timothy Lu, an MIT bioengineer, might have a way to get answers faster.

Lu originally trained as a computer scientist, but developments in genetics and genetic engineering, including the Human Genome Project, prompted him to take his career in a new direction.

CREDIT: JEFF FITLOW/RICE UNIVERSITY

Jeffrey Tabor and Prabha Ramakrishnan engineer* E. coli *with two-component systems taken from other bacteria to enable them to sense and respond to different stimuli.

"The idea that you could think about DNA as a kind of code was really appealing," said Lu. "So, I switched fields and jumped into the field of synthetic biology. And it's been fun ever since trying to figure out how to program cells to do different things."

During his early work on bacterial biosensors for the gut, Lu recognized some inherent shortcomings. While bacteria are easy to administer, said Lu, "the downside is that it's hard to get more granular detail about where you are in the gut and what's going on in real time. You have to wait for the bacteria to come out the other end. Bacteria aren't good at communicating over long distances. So, we had to figure out how to combine these together with some other modality, and electronics made a lot of sense."

Lu teamed up with MIT electrical engineer Anantha Chandrakasan to see what a partnership between bacteria and electronics could achieve. In 2018, this pair debuted a bioelectronic sensor that detects bleeding in the gut (6). Lu and his team engineered a bacterium to produce luminescence in response to heme, which is present in blood. They packaged these bacteria with an electronic sensor to measure the luminescence output and wirelessly transmit this information to a computer or smartphone. In tests, this bioelectronic package detected blood in pigs' gastrointestinal tracts in near real time.

Next, Lu wanted to make a few upgrades. First of all, at more than 3 centimeters long, the original device was too big for human use. With the help of fellow engineers Rabia Yazicigil of Boston University and Giovanni Traverso of MIT, he shrunk the capsule down to less than 1.4 cm in length, smaller than a typical pill camera. In work described in a preprint, the researchers also created a multifunction sensor with different types of bacteria that report on four different markers associated with inflammatory bowel disease (7). In the future, Lu envisions a sensor with a dozen or more types of engineered bacteria to sense different chemicals in the gut for even greater precision when diagnosing and monitoring disease.

While many researchers, including Tabor and Lu, focused on markers of gut inflammation, this certainly isn't the only application. Jeff Hasty's team at the University of California, San Diego showed in a preprint that engineered bacteria also detect colorectal cancer in mice (8).

Platt said that unraveling the mystery of what's happening in the gut is likely important for a slew of diseases. "There's so much we don't know about the human intestine, but we know that it's really, really, important. It's connected to all sorts of diseases from diabetes to inflammatory bowel disease. It can even change your behavior."

Whether packaged in hydrogels or working in concert with miniature electronics, whether sensing one environmental factor or many, scientists hope that their approaches will not only enable us to understand gut processes more fully, but also provide diagnostics and monitoring to help inform management of life altering diseases. ■

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Don't Hold Your Breath

Seeking an accessible diagnostic medium, researchers are rooting through the concoction of compounds in exhaled breath vapor for the metabolic manifestations of disease.

Ongoing advances in biomarker discovery and detection enable scientists to realize the diagnostic value of exhaled breath vapor.

BY SARAH ANDERSON, PHD

PAUL THOMAS, AN ANALYTICAL chemist at the breath diagnostics company Bioxhale, concluded his presentation at the 2020 Breath Biopsy Conference with a cartoon by Gary Larson. The cartoon features two beavers gnawing at a lumberjack's wooden leg. One beaver says to the other, "Hey! They're edible! This changes everything!"

"The point is that we jump to conclusions so readily based on such limited observations and data," Thomas said. "It's just a warning to anybody who's listening to me that I could be that beaver, wildly mistaken and misinformed about the nature of my observations, and that some humility and skepticism is entirely appropriate in all of these things."

This sage sentiment, while applicable to scientific research in general, is especially relevant to the pursuit of methods to detect disease in exhaled breath. The notion that breath might provide an unexpected source of health information can be traced as far back as the Greek physician Hippocrates, who sniffed out liver disease in patients with fishy-smelling breath (1).

Translating this ancient anecdotal practice to a rigorous modern diagnostic tool, however, has proven challenging. To pinpoint unique features in complex breath samples that can be reliably traced to disease, researchers are exploring new directions in biomarker discovery, data processing, and breath sampling workflow. In doing so, they aim to finally unleash breath's diagnostic potential, making detection of conditions ranging from COVID-19 to cancer as easy as breathing.

Just breathe

In a long line of biofluids that researchers have explored as diagnostic media — including blood, sweat, tears, and urine — the appeal of targeting the involuntary, non-stop process of breathing is clear. Collecting breath is completely noninvasive, and there's no limit to how much a person can provide.

> "Anything that can escape into the breath and that we can measure can tell us a lot about the state of health or disease of an individual."
>
> *– Raed Dweik, Cleveland Clinic*

It turns out that Hippocrates was onto something when it comes to breath's diagnostic value. As diseases disrupt key biochemical pathways and metabolic processes, they leave behind a trail of molecular markers in their wake, and those that evaporate end up in exhaled breath vapor. These volatile organic compounds (VOCs) are produced directly within the respiratory system or gastrointestinal tract or carried to the lungs from circulating blood, enabling the composition of exhaled breath to indicate distress throughout the body. "Anything that can escape into the breath and that we can measure can tell us a lot about the state of health or disease of an individual," said Raed Dweik, a pulmonologist and breath diagnostics researcher at the Cleveland Clinic.

Breath-based signals of disease aren't blaring sirens that sound out of dead silence, though. "Almost all the biomarkers we find in breath exist in [healthy] people. It's just much higher or much lower in a disease state," Dweik said. Just as blood tests measure the concentrations of certain chemicals relative to a normal range, breath testing looks for changes in the levels of VOCs.

These changes can be challenging to detect in the rich mixture of breath, where thousands of VOCs comingle at concentrations comparable to a single spoonful of sugar in an Olympic-sized swimming pool. In addition to the many endogenous sources, external substances yield additional compounds in exhaled breath that can interfere with the detection of biomarkers of disease. As anyone who's ever eaten garlic knows, ingested foods leave their own mark on the breath. Similarly, "As we walk around or drive around, we are sadly the vessels. Everything in the environment that we breathe in, we eventually breathe out," Dweik said. "All these confounders I think need to be solved for to look for the morsel of information we want about the health of the metabolic state."

Mining for morsels

Thomas's career in breath research originally focused on detecting the environmental toxins that people breath in and out. But when his project measuring radiation exposure was derailed by the COVID-19 pandemic, he wondered if the SARS-CoV-2 virus might create its own VOC signature in the breath. To see if they could distinguish COVID-19 from other diseases, his team collected breath samples from patients diagnosed with COVID-19 as well as asthma, chronic obstructive pulmonary disease, bacterial pneumonia, and cardiac conditions. The researchers analyzed the breath samples using a technique that separates the various

VOC components and detects each one via its movement through a gaseous electric field. Using statistical methods, they developed an equation that provides a COVID-19 breath score based on the levels of six VOCs and found that it could predict infection status with about 80 percent accuracy (2).

The ability to differentiate COVID-19 from other respiratory diseases comes from looking at the virus's systemic effects beyond the lungs, according to Thomas. "The reason it works is that COVID is such a powerful disease; it affects just about everything in your body," he said. For example, the team detected high levels of aldehyde compounds, which are linked to oxidative damage and inflammation, high amounts of ketone metabolites, indicating irregular energy metabolism, and a low concentration of methanol, suggesting disrupted activity in the gut microbiome. "When you put all of those effects together, you get a pattern of chemicals in breath that changes quite significantly because of all of this insult and dysregulation in someone who's ill with this disease," Thomas said.

Since Thomas's 2020 study, the delta and omicron variants emerged, bringing with them not only spikes in cases, but potential changes in the COVID-19 VOC profile. Cristina Davis, an interdisciplinary engineer at the University of California, Davis, wondered whether variants may lead to unique changes in the constellation of VOCs in breath. Her team collected breath samples from healthy people and those with COVID-19 during the delta wave (through November 24, 2021) and the omicron wave (after January 11, 2022).

They analyzed the samples using a technique that concentrates the VOCs onto a solid adsorbent surface, applies heat to dislodge the compounds, and separates and identifies each one by its mass. To control for environmental confounders, the researchers compare their human samples to environmental samples and delete any ambient compounds from the dataset. They also ensure that participants haven't ingested anything for at least an hour prior to giving a breath sample to minimize detection of ingested VOCs.

Davis's team measured the abundance of hundreds of VOCs in the breath samples and developed statistical models to predict COVID-19 infection using data from all of the COVID-19 patients versus just those with the delta or omicron variants (3). They found that the models developed for each specific variant showed higher accuracy than their general COVID-19 model.

"In the volatile organic compound profiles, there were some pieces in common, but part of it shifted during the omicron strain that proliferated," Davis said. "That's something we need to be aware of because we may need to rebuild those models if you get a big enough variant shift." The researchers are currently evaluating their ability to distinguish COVID-19 in all its forms from the flu now that flu cases have reemerged from a two-year lull due to social distancing, masking, and hand washing.

Davis's team also examines VOCs emitted by cultured cells to complement their measurements in exhaled human breath, helping them tie their biomarkers back to biochemical mechanisms (4). "When you're using cell culture studies, you can actually understand those signal transduction pathways and where the biology is and where it's coming from so that it's not just phenomenology that you're measuring," Davis said.

Breath-based diagnostic tests could have a major impact on reducing transmission of a variety of viruses. Davis's COVID-19 study relied on symptomatic participants, but she is interested in investigating if VOC analysis can also pinpoint asymptomatic infections — something her cell culture studies predict is possible. "We're looking at what we believe is the host response to infection rather than looking for the virus itself, which may or may not have enough copies for the PCR tests to actually detect it yet," she said. "The host response to infection starts as soon as infection starts, and so the question is, based on that cell culture data, is there a point at which we can measure those biomarkers before the traditional measures would be available?"

> "The ultimate goal for breath testing is to be like the breathalyzer for alcohol. If you can do it on the side of the street, you can do it anywhere else."
>
> *– Raed Dweik, Cleveland Clinic*

Thomas sees COVID-19 as a case study for how breath tests can protect underserved populations that don't have access to traditional testing technology. His VOC detection instrument is relatively portable at about the size of a printer, but "the ultimate goal for breath testing is to be like the breathalyzer for alcohol," Dweik said. "If you can do it on the side of the street, you can do it anywhere else."

Once researchers have identified the critical VOC biomarkers of a disease, small sensors that measure specific compounds could translate their diagnostic discoveries to point of care applications. "Being able to know what compounds are in the breath in a handheld device would be very powerful," Dweik said. "That will require collaboration between scientists, physicians, technicians, and analysts."

A pattern of disease

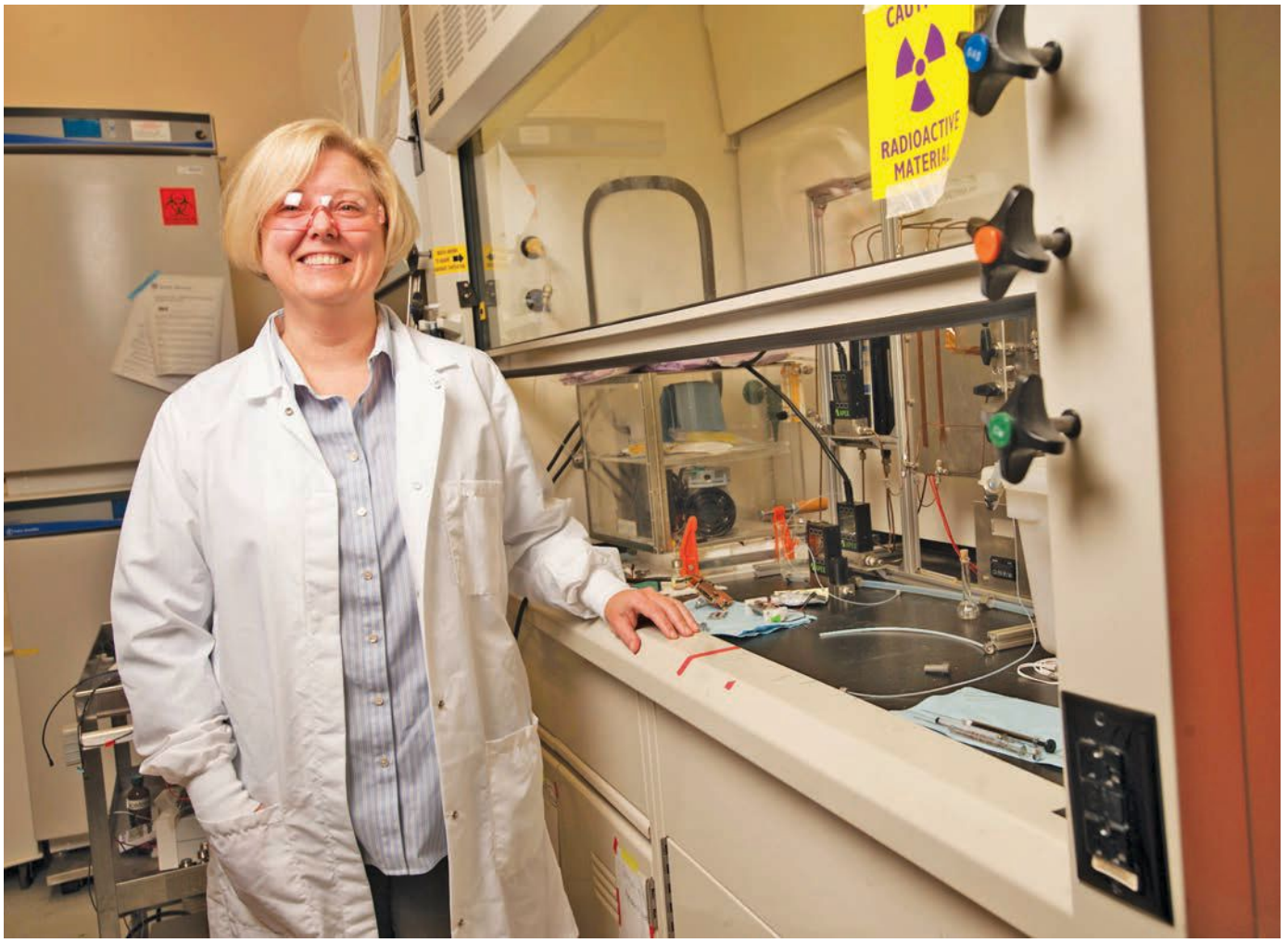
While breath testing technology might not need to be quite so miniaturized for applications in diseases such as cancer, it could provide a more accessible screening tool for primary care settings. "We envision you could just walk down the street to either your general practitioner's office or to a pharmacy and give a breath sample that way," said Stephen Graham, a pharmacist and CEO of the breath diagnostics company Breathe BioMedical. Breath testing may even be able to pick up on early metabolic changes that signal cancer before traditional imaging techniques would detect a tumor.

Breathe BioMedical's sampling device lets breath from the upper respiratory system pass through before collecting alveolar breath from deep in the lungs, minimizing the presence of ingested compounds in the sample. VOCs in the alveolar breath are trapped onto a solid adsorbent surface, liberated using heat, and injected into an optical cavity with super reflective mirrors on either side. As laser light bounces off the mirrors about 1,000 times, the VOCs absorb more and more of the light with each pass. This highly sensitive technique generates a spectrum of an individual's breath sample that captures minute shifts in VOC abundance. Acquiring these breath spectra from a group of individuals with the same disease provides a distinct breath print of the disease.

Researchers at Breathe BioMedical use machine learning algorithms to analyze the complex spectra and identify distinguishing features for a disease, accelerating the development of diagnostic models from a collection of breath samples. "We don't look at exact concentrations of specific VOCs," Graham said. Rather, "The model learns, and it says, 'Okay, these are the features that look like cancer, and these are the features that look like noncancer.'" Graham compares this approach to disease sniffing dogs. "The dog doesn't smell cancer and say, 'Oh, yes, I smell so many parts per billion of isoprene,'" he said. "It just recognizes that the features of that smell are what cancer is."

It's easy to lose sight of metabolic underpinnings in a mountain of mysterious peaks in a spectrum, however. Dweik recalled an incident in which his team uncovered a data feature that appeared to give perfect diagnostic accuracy — "almost too good to be true," he said. Indeed, when they investigated the compound behind the feature, they discovered that it arose from a VOC in the handwash at the hospital where the disease cohort was treated. "You could fall into the trap of thinking you found the test, but really, it's a contaminant," Dweik said.

The Breathe BioMedical team compares every breath spectrum to a sample of room air to control for potential ambient confounders. They can also process their spectra to identify and quantify specific VOCs,



Cristina Davis investigates how infectious respiratory diseases such as COVID-19 modify exhaled breath.

CREDIT: GREGORY URQUIAGA/UNIVERSITY OF CALIFORNIA, DAVIS

enabling them to confirm that they are measuring biologically relevant compounds.

In a recent study, the researchers collected breath spectra from healthy people and those with lung cancer and analyzed them using both breath print feature recognition with machine learning and standard VOC composition analysis (5). They observed that the breath print method gave better diagnostic accuracy. While researchers have struggled to define a common VOC profile in different individuals with lung cancer, machine learning can reveal subtler features of the disease across the entire spectrum. "We think that we can solve some of that problem with heterogeneity by taking a more comprehensive approach to looking at the data through the absorption," Graham said.

Analyzing a breath sample comprehensively may also enable it to provide a general snapshot of health rather than a test for one specific disease. "Our vision is that 10 years from now, you'll be able to go in, give a breath sample, and then receive a panel of screening tests for a number of different diseases from the same breath sample," Graham said.

As the team continues to validate their method against more traditional biomarker discovery approaches, Graham hopes that spectral feature recognition using machine learning will become the standard in the breath diagnostic space. "It's a shift in thinking, and I think we're the first ones who are really spearheading that shift," he said.

CREDIT: BREATHE BIOMEDICAL

Stephen Graham and his team at Breathe BioMedical have pioneered the use of machine learning algorithms to analyze distinct breath prints of disease.

CREDIT: OWLSTONE MEDICAL

Billy Boyle and fellow researchers at Owlstone Medical developed exogenous compounds that target unique metabolic pathways in lung cancer and other conditions, yielding specific disease markers in exhaled breath.

> "Often, it comes down to getting the basics right to ensure you are detecting chemicals that are truly coming from breath, understand what they are, understand what the ranges of those compounds are in the healthy population, and then think about applying it to the disease population."
>
> *– Billy Boyle, Owlstone Medical*

Probing deeper

The inability to identify a VOC signature for lung cancer may also stem from the fact that many of the compounds result from nonspecific responses to disease. "If you look at the altered metabolism of lung cancer and at some of the processes that relate to the production of VOCs, things like oxidative stress and inflammation, you're probably not going to get the specificity that you would need for a test for something like early cancer detection," said Billy Boyle, an engineer and CEO of the breath diagnostics company Owlstone Medical.

Boyle wondered if simply measuring the endogenous VOCs in the body that end up in exhaled breath was the best strategy for complex diseases such as cancer. "Part of it was from the frustration that there's got to be a better way if we take a more bottom-up type approach to understand the biology and try to target specific pathways," he said.

A breakthrough came from a compound called limonene, which is found in citrus. "We would see it pop up all the time in our analysis, and we would discount it and say, 'Oh, limonene just comes from diet,'" Boyle said. However, when the team explored the journey limonene takes in the body, they discovered that it's metabolized by liver enzymes (6). "Compounds that we previously thought, 'they're exogenous, so therefore they're probably chemical noise'— what if we introduced them into the body deliberately?'" Boyle said. "What if we start to think about the exogenous compounds as being probes for particular pathways?"

In searching for unique characteristics of cancer they could target with an external compound, the researchers came across extracellular β-glucuronidase enzymes in the environment of solid tumors, such as those found in lung cancer. They designed a deuterium isotope-labeled molecular probe to administer into the body, where any β-glucuronidase enzymes at the tumor site would cleave a bond, yielding deuterated D5-ethanol that comes out in exhaled breath (7). This product can be detected by its heavier mass, which distinguishes it from ethanol in exhaled breath due to alcohol consumption, providing an unambiguous VOC marker of cancer. "The hope is you'd see almost this very clear binary signal of the presence of D5-ethanol in breath, signifying the glucuronidase and the tumor microenvironment," Boyle said.

Administering the exogenous probe by inhalation enables it to specifically detect lung tumors over other types of solid tumors and gastrointestinal bacteria that also produce β-glucuronidase, said Anil Modak, a medicinal and pharmaceutical chemist and scientific advisor to Owlstone Medical. Delivering exogenous probes through inhalation or ingestion rather than intravenous injection is also critical for upholding the basic tenant of breath diagnostics as a noninvasive platform, he added.

The Owlstone Medical researchers found that β-glucuronidase is present in the tumor environment of 90 percent of early-stage lung cancer tissue samples. However, in its first stages, "the tumor is extremely small and the amount of β-glucuronidase enzyme increase is very minimal, so the amount of D5-ethanol that is produced may be almost at undetectable levels. …Can that D5-ethanol be detected at very small amounts in exhaled breath?" Modak said. "The clinical unmet need is identifying the lung cancer prior to it being detected by a CT scan or PET scan."

Boyle aims to boost sensitivity by introducing an excess of the exogenous probe, allowing one enzyme to cleave multiple substrates into product, and by extending the breath collection period to enrich the sample. He is deeply aware of the need for early cancer detection, having lost his wife to colon cancer that was diagnosed at a late stage. "I really understood why early detection matters in terms of survival outcome," Boyle said. "That was a key moment for me to say I know what I want to do with the rest of my life, which is to try to hopefully find ways to develop tests that pick up disease sooner."

While Boyle is blazing a new frontier in early cancer detection with exogenous probes, he is also honing the fundamentals of breath diagnostics for other diseases using endogenous VOCs. "Often, it comes down to getting the basics right to ensure you are detecting chemicals that are truly coming from breath, understand what they are, understand what the ranges of those compounds are in the healthy population, and then think about applying it to the disease population," he said.

Toward this end, the Owlstone Medical team compiled the Breath Biopsy® VOC Atlas documenting the concentration ranges of approximately 150 compounds in the exhaled breath of a heterogeneous group of healthy people (8). The goal of this catalog is to help researchers establish a normal VOC baseline, separate biomarkers from confounding compounds, and ultimately draw well informed conclusions that don't leave them vulnerable to the beaver trap. "This will be a growing resource over time," Boyle said. "That's something that we're investing in doing, but with a view to make it available to the broader community." ■

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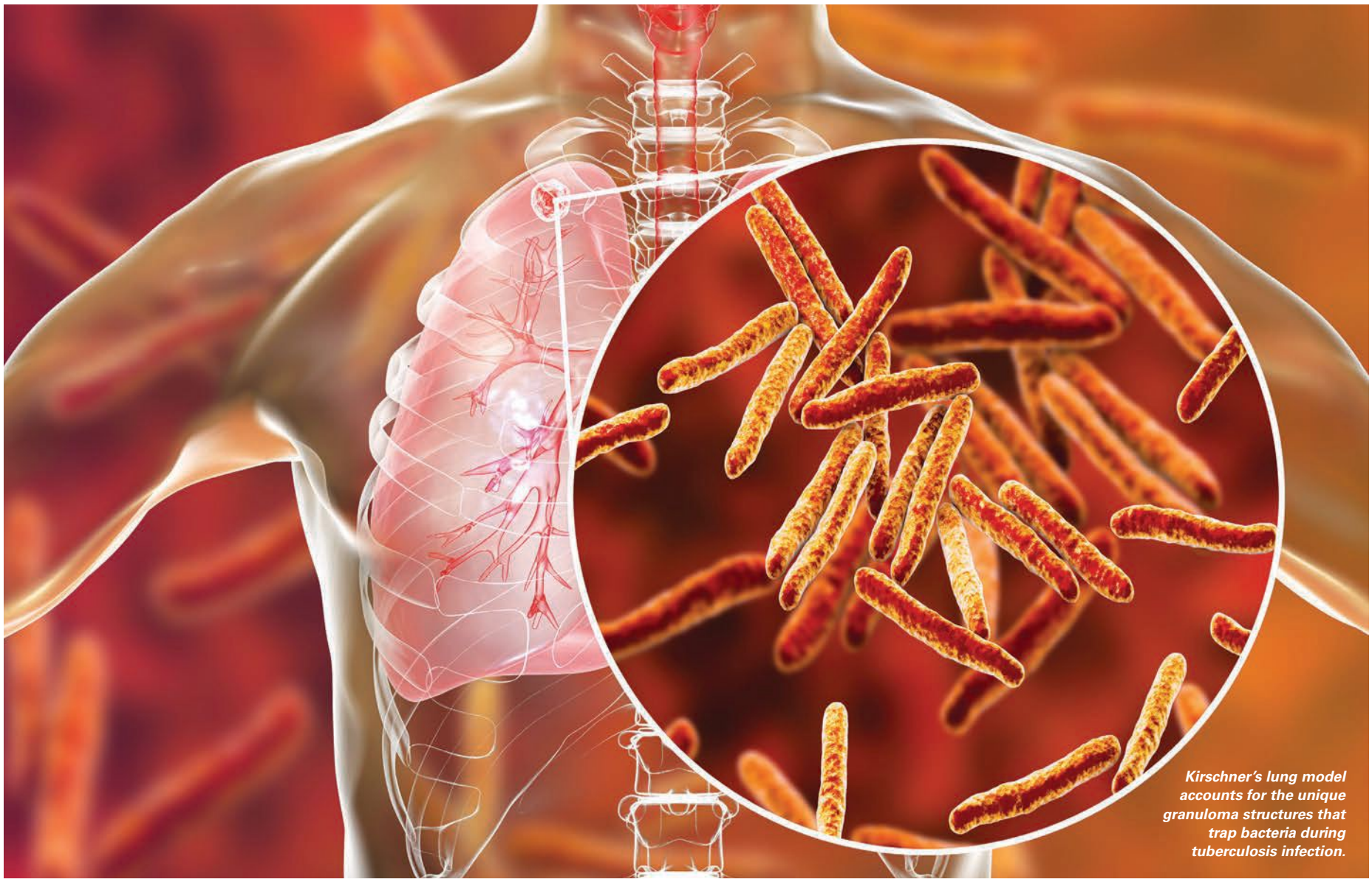
Calculating Better Tuberculosis Treatments

Denise Kirschner developed an equation-based lung model of a fatal but forgotten infectious disease, revealing an unexpected role for math in drug discovery.



Kirschner's lung model accounts for the unique granuloma structures that trap bacteria during tuberculosis infection.

INTERVIEWED BY SARAH ANDERSON, PHD

DENISE KIRSCHNER thought she needed to become a doctor to help sick people. But as she discovered an aptitude for mathematics throughout high school and college, she wondered whether math might provide an underexplored route toward treating disease. She found her answer during a summer research program at Los Alamos National Laboratory where she used mathematical modeling to study the dynamic interactions of the HIV virus within the body. "I was hooked," Kirschner said. "I thought, this is what I'm supposed to do because I'm really good at math, but I love biology. So, it's a perfect marriage for me."

Now the director of the Computational Immunobiology of Tuberculosis Lab at the University of Michigan, Kirschner continues to take the road less traveled, focusing on an overlooked bacterial disease. "If you ask anybody in the street about tuberculosis, they'll say, 'Oh, that was in the 1800s, right?'" she said. In reality, tuberculosis currently infects almost two billion people — one fourth of the world's population — with more than 80 percent of cases and deaths concentrated in low and middle income countries (1,2).

The World Health Organization and United Nations declared March 24 World Tuberculosis Day, marking the date in 1882 on which microbiologist Robert Koch discovered the *Mycobacterium tuberculosis* pathogen, aiming to raise public awareness and drive research efforts to eradicate the disease.

Kirschner has the same goal. By creating mathematical equations that represent the biological processes at play during pulmonary tuberculosis infection, she developed a novel computational lung model of the disease to expedite the search for new treatments. Kirschner can use her virtual lung to screen antibiotic and vaccine regimens and predict the most promising ones, providing a valuable complement to gold standard large animal models of tuberculosis. What's more, her simulated lung is part of a larger body of computational organ models that together could revolutionize early-stage drug development.

What are the limitations of existing tuberculosis treatments?

The tuberculosis pathogen has existed since the time of the Egyptian mummies, and yet we still don't know how to effectively combat it. The current vaccine for tuberculosis consists of a live bacteria that is a weaker cousin of the tuberculosis pathogen that is intended to train the immune response to infection. However, this vaccine was developed about 100 years ago, and its efficacy falls somewhere between zero and 80 percent.

> "If you ask anybody in the street about tuberculosis, they'll say, 'Oh, that was in the 1800s, right?'"
>
> *– Denise Kirschner, University of Michigan*

The vaccine is generally not administered in the United States and United Kingdom because the efficacy is so low and because it interferes with the skin test for tuberculosis. There is a drug regimen that can squelch the disease, but it requires people to take four antibiotics that can cause side effects such as vomiting and diarrhea for six to nine months. Most people don't even finish a 10-day course of a single antibiotic. Getting people to comply with this treatment is a major challenge, and if they don't, drug resistance develops rapidly.

How can computational models help develop better drugs for tuberculosis?

The hallmark of tuberculosis is lung granulomas, which are dense structures similar to tumors. They are made up of the bacteria and various immune cells. Drugs need to reach the granuloma, penetrate it, and find the bacteria, many of which are trapped in necrotic areas deep in the granuloma core. There isn't a good small animal model for this system; mice don't get granulomas in their lungs. Rabbits do, but no immunoreagents to study rabbit granulomas have been developed. Researchers use nonhuman primates as tuberculosis models, but they are very expensive to acquire and maintain. It's virtually impossible to get 50 monkeys for a study.

What computational modeling brings to the table is the ability to narrow the design space. Just 10 drug candidates in different combinations, doses, and time courses can yield 10^{17} possible regimens, which is too many even for a computer to handle, so we still need to optimize the regimen design parameters up front. But essentially, we can say that these are the most promising drug regimens within that giant space, so here's where the experimentalists should focus.

How did you develop your computational lung model of tuberculosis?

We wanted to model the immune response to understand how a drug affects the dynamics between all of the relevant cells and not only the bacteria. We studied the literature and worked with collaborators to gather everything known about the immune response to tuberculosis. We wrote it all down in equations that track the physical and chemical interactions between various components over time and space. This method examines the average behavior of the entire population of T cells, macrophages, bacteria, and so on. We then moved from equation-based modeling to computer algorithm-based modeling, in which we translate those equations into computer code, such as "if-then" statements, for each individual species. For example, if a T cell meets a macrophage, then it can secrete a cytokine that activates that macrophage to kill bacteria.

To develop a whole lung model for pulmonary tuberculosis, we expanded this approach to include the lungs, which serve as the site of the disease, the lymph nodes, which produce

CREDIT: A. THOMASON/UNIVERSITY OF MICHIGAN

Denise Kirschner developed a mathematical model of pulmonary tuberculosis to improve the efficiency of drug discovery.

immune cells, and the blood, which carries immune cells back and forth from the lymph nodes to the lungs (3). We developed a system of equations representing the processes occurring within the lung granulomas, the lymph nodes, and the blood as well as the dynamics as immune cells move between them.

How can you use this model to screen drug regimens for tuberculosis?

Our collaborators evaluate tuberculosis drugs in nonhuman primate and rabbit models and provide data on the drug's pharmacokinetic and pharmacodynamic properties and, for the primates, its effect on the granulomas. We use some of those datasets to calibrate the model and then test it against separate datasets to confirm that when we input information about a drug's mechanism, the model gives the correct output in terms of the host response.

> "There are about 20 of us in the computational world who have built these types of models in various organs, and our goal is to sew them all together to build a digital human twin."
>
> *– Denise Kirschner, University of Michigan*

Once we've validated the model in this way, we can use it to explore many questions about drug regimens. For example, we recently used the model to screen different regimens of standard and new tuberculosis antibiotics and identified those that eliminated the bacteria the fastest, which we predicted to be the most effective. Our collaborator then tested the drugs in nonhuman primates and observed that we had successfully predicted the regimens that performed the best *in vivo*.

How adaptable is your model to other organs?

It is lung specific, but we could adapt it to study other respiratory diseases such as lung cancer, chronic obstructive pulmonary disease, and COVID-19. I want to be the lung person and work with other researchers who are the kidney or heart or brain people. There are about 20 of us in the computational world who have built these types of models in various organs, and our goal is to sew them all together to build a digital human twin. When Ford built the first car, it was a clunker, but it drove, and now we have the Mercedes-Benz. Similarly, we'll start with a Model T and build our way up. We could link the different components together with some nuts and bolts, and over time, turn our digital twin into a Mercedes. ■

This interview has been condensed and edited for clarity.

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infectious disease

Goo to the Rescue

Sugars and proteins in mucus help protect us from infection in complex ways.

Glycoproteins in mucus can protect us from infections.

BY HANNAH THOMASY, PHD

IT MIGHT NOT SEEM LIKE CORALS and humans have much in common. Corals can't see or hear; they don't have brains or bones or lungs or hearts. Just like humans, though, corals need to protect their soft tissues from a world full of microbial invaders. Both species, and indeed, most animal species on planet Earth, have a similar first line of defense: thick, slimy mucus (1).

The fact that mucus exists in some of humanity's most distant relatives corroborates its ancient origins, yet this sticky goo remains underappreciated and poorly understood. "People really thought that mucus was a waste product for a very long time," said Jessica Kramer, a biomedical engineer at the University of Utah.

In recent years, scientists have begun to appreciate mucus. Often, it protects us from harmful infections, but in some cases, it may actually increase our vulnerability. Learning more about these processes and how they are regulated could yield important benefits.

"With antibiotic resistance becoming a threat to our current ways of treating infections, I think it is imperative to find alternative treatments," Sara Lindén, a microbiologist at the University of Gothenburg, wrote. "Enhancing and mimicking the body's natural defenses, such as the mucin-based defenses, appear to be plausible paths forward."

Know your mucus

Mucus protects the delicate epithelial tissues of our eyes, digestive systems, respiratory systems, and urogenital tracts. It is a highly heterogenous substance. "The structure is unique to each species and actually varies between different people," said Kramer. "Even on one person's bodily surfaces like the eyes versus the lungs, or the reproductive tract versus the lungs, the composition of the mucus is different."

> "People really thought that mucus was a waste product for a very long time."
>
> *– Jessica Kramer, University of Utah*

Mucus is largely composed of mucins, macromolecules with protein backbones and sugars called glycans sticking out in all directions like bottlebrushes. A single mucin can have more than 200 different types of glycan structures, making mucins difficult to study and recreate in the lab (2).

Our internal surfaces are generally covered with two distinct layers of mucins. "There's the mucus that everybody is quite familiar with that's made of mucins that have been secreted by cells. They are freely floating; we blow them into a tissue," said Kramer. "However, I think most people are unaware of a structure called the glycocalyx, which is a layer of mucins that are actually attached to our cell surfaces." In order to infect a cell, pathogens have to penetrate the layer of free floating mucins as well as the transmembrane mucins of the glycocalyx.

Infection protection

In a recent preprint, Karin Strijbis, an infection biologist at Utrecht University, reported the role of a transmembrane mucin called MUC1. In the course of her research, she incubated a layer of MUC1-expressing lung cells with the SARS-CoV-2 virus. She removed the MUC1 layer from a different set of lung cells before adding virus to these cells as well. Without MUC1 present, the SARS-CoV-2 virus ran amok, infecting far more cells than the unaltered population.

"The mucin is making a barrier that prevents the virus from reaching its receptor," said Strijbis. "Mucins are very large, so they're sticking out quite far from the membrane. And the receptor that the virus needs to bind to is much closer to the membrane. The mucin is like a little umbrella that's just there in between the virus and the receptor that prevents it from connecting. If you cleave off the whole umbrella, then the virus can reach the ACE2 receptor and enter the cell."

Mucins can run interference in other ways as well. One example is mucins' interaction with *Helicobacter pylori*, a relatively common bacteria that occasionally causes gastritis, stomach ulcers, and even stomach cancer. Early work showed that MUC1 protected against *H. pylori* infection in mice; without MUC1, mice had higher bacterial loads in their stomach linings and more severe gastritis (5).

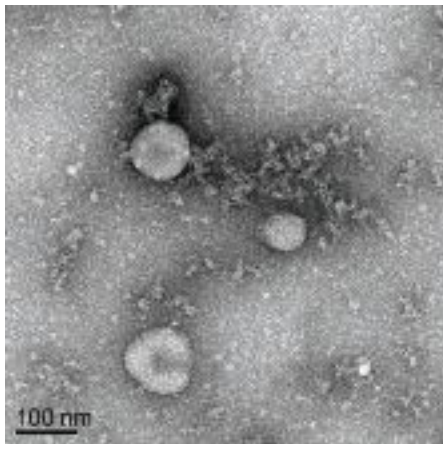
Lindén showed that MUC1 helps protect host cells by tricking the bacteria. In order to infect cells, bacteria need to attach themselves to the cell, which they do with proteins called adhesins. Lindén demonstrated that *H. pylori* bound to MUC1 using these adhesins and that once this binding occurred, the extracellular part of the mucin detached from the cell.

"Mucins can act as releasable decoys, thereby hindering attachment to the epithelial surface," she explained. "Mucins that have strong abilities to bind to *Helicobacter pylori* lead to fewer bacteria reaching the epithelial surface since mucin-bound *H. pylori* gets shed from the mucosal surface and transported away from the stomach with the gastric emptying."

Mucins also appear to regulate microbial behavior in complex and poorly understood ways. One of the microbes that mucins manipulate is *Candida albicans*. This single cell fungus usually lives harmlessly on human mucus membranes, but it occasionally causes bothersome infections such as thrush or a life-threatening infection known as invasive candidiasis, which kills thousands every year (6). Rachel Hevey, a biochemist at the University of Basel, along with collaborators at the Massachusetts Institute of Technology (MIT), showed that mucus-associated glycans may be key for keeping this microbe tame.

"When *Candida* starts to form filaments, this is associated with its pathogenic form," said Hevey. "Filaments allow it to attach to surfaces like epithelial surfaces and to penetrate and establish infections. The glycans prevent them from being able to form these filaments. So, they stay in what we call a yeast form, but it's essentially just a round form with no filaments. Then it can't really establish an infection as easily. The glycans force it to stay in a healthy form and not the pathogenic form."

The ability of mucins to manipulate the behavior of pathogens is not limited to *C. albicans*. When *Pseudomonas aeruginosa* bacteria, which can cause deadly lung and blood infections, mix with mucins, they reduced the expression of virulence genes, including those related to toxin secretion (7). Mucins in saliva suppress pathways related to quorum sensing, the means by which bacteria communicate with each other and coordinate behaviors, including those that promote infection (8).



CREDIT: CASIA WARDZALA, KRAMER LAB

Transmission electron microscopy shows how mucins bind to human coronavirus OC43.

Mucin-microbe interactions are not only related to infection, but may also influence the transmissibility of the pathogen (9). For example, some viruses can spread through contact with contaminated objects or surfaces. Kramer showed that a virus related to SARS-CoV-2 was still highly infectious after virus-laden droplets of saline dried on a surface. But when these droplets also contained mucins, as droplets expelled during a cough or sneeze would, the virus's ability to infect cells was dramatically impaired, indicating that mucins likely reduce viral transmission through touching contaminated surfaces for at least some types of viruses (9).

> "The glycans force it to stay in a healthy form and not the pathogenic form."
>
> *– Rachel Hevey, University of Basel*

Not all mucins are created equal. A study of 28 people identified more than 600 gastric mucin-associated glycans with only six shared by all participants. These different glycosylated mucins also seem to vary in their interactions with pathogens (10).

More research into this field, "could improve our understanding of how people's compositions of mucus affect their vulnerability to contracting serious disease," Kramer said. "Of course, there are many other factors, such as comorbidities and immune function and all that, but it could be one piece of the puzzle. And then, on the flip side, it might be helpful for identifying who is more likely to spread the virus."

Mucin-inspired therapeutics

Mucins could do much more than help assess risk, however. Many researchers look at mucin manipulation or mimicry as a way to prevent or treat disease. One strategy is to develop ways to increase production of specific mucins but, said Strijbis, this needs to be done carefully.

For example, MUC1seems to protect against SARS-CoV-2. On the other hand, overproduction of secreted mucins associates with worse COVID-19 outcomes. MUC1 overexpression also associates with some forms of cancer (11). "There's always a fine line with mucins," Strijbis said.

A ready made way to manipulate mucins may already exist. Just as mucins manipulate microbes, some microbes alter the composition of mucus (12). Strijbis and her team are currently screening a library of probiotic bacteria to determine their effects on mucins. "We want to find out how good bacteria can stimulate healthy mucosal barriers and see how we then can prevent pathogen invasion."

Other researchers want to create drugs based on mucins or elements of the mucins. A team at the University of Copenhagen genetically engineered cells to produce artificial mucins and is now exploring the interactions of these mucins with bacteria and viruses (13). Researchers at MIT have created mucin-inspired polymers that bind to cholera toxin (14).

Hevey thinks that it might not be necessary to mimic the entire glycoprotein, which is large and complex. Her team studies the glycans. The unattached glycan molecules aren't very good as drugs, said Hevey, since they get excreted too quickly, but they may be valuable for informing drug design.

"A big focus of our research is understanding the chemical properties of the molecule and which are important for biological activity and which aren't," she said. "And then [we use] our synthetic methodologies to make molecules that have better drug-like properties but still mimic the natural activity of the glycan."

An evolutionary arms race

As mucins evolved to protect us against pathogens, the pathogens also evolved. Some came up with ways to overcome mucin-mediated defenses. For example, some mucins bind to viruses, using their glycans like a sponge to mop them up.

"But the flu virus is really sneaky," Kramer said. "It has co-evolved mechanisms to chew off those sugars, releasing itself from the mucin."

Some microbes go beyond simply evading mucin-based defenses; they evolved ways to use mucins to their advantage. In a study of the pathogen *Salmonella enterica*, for example, Strijbis found that bacteria exploited the normally protective MUC1 and used it to help them enter the cell. Removing MUC1 reduced bacterial invasion of gut epithelial cells (15).

More research is needed to determine the

CREDIT: STEFAN BÄCHTOLD

Rachel Hevey studies how glycans interact with potential pathogens.

CREDIT: DAN HIXSON, UNIVERSITY OF UTAH

Jessica Kramer studies the effects of mucins on coronaviruses and is developing synthetic mucus.

> "The flu virus is really sneaky."
>
> *– Jessica Kramer, University of Utah*

role of mucins in mediating susceptibility to different infectious agents, and this will influence scientists when designing mucin-inspired therapeutics.

Into the future

People often ask Kramer how to make their mucus more protective. While genetics are important, factors like diet and environment may also affect mucus composition. Unfortunately, there simply isn't enough evidence yet to make solid recommendations about how to optimize mucus-mediated protection.

However, as researchers begin to unravel the complex contributions of mucus, therapeutics targeting or inspired by this often-overlooked substance may help give humanity the upper hand in the constant battle against microbial adversaries. ■

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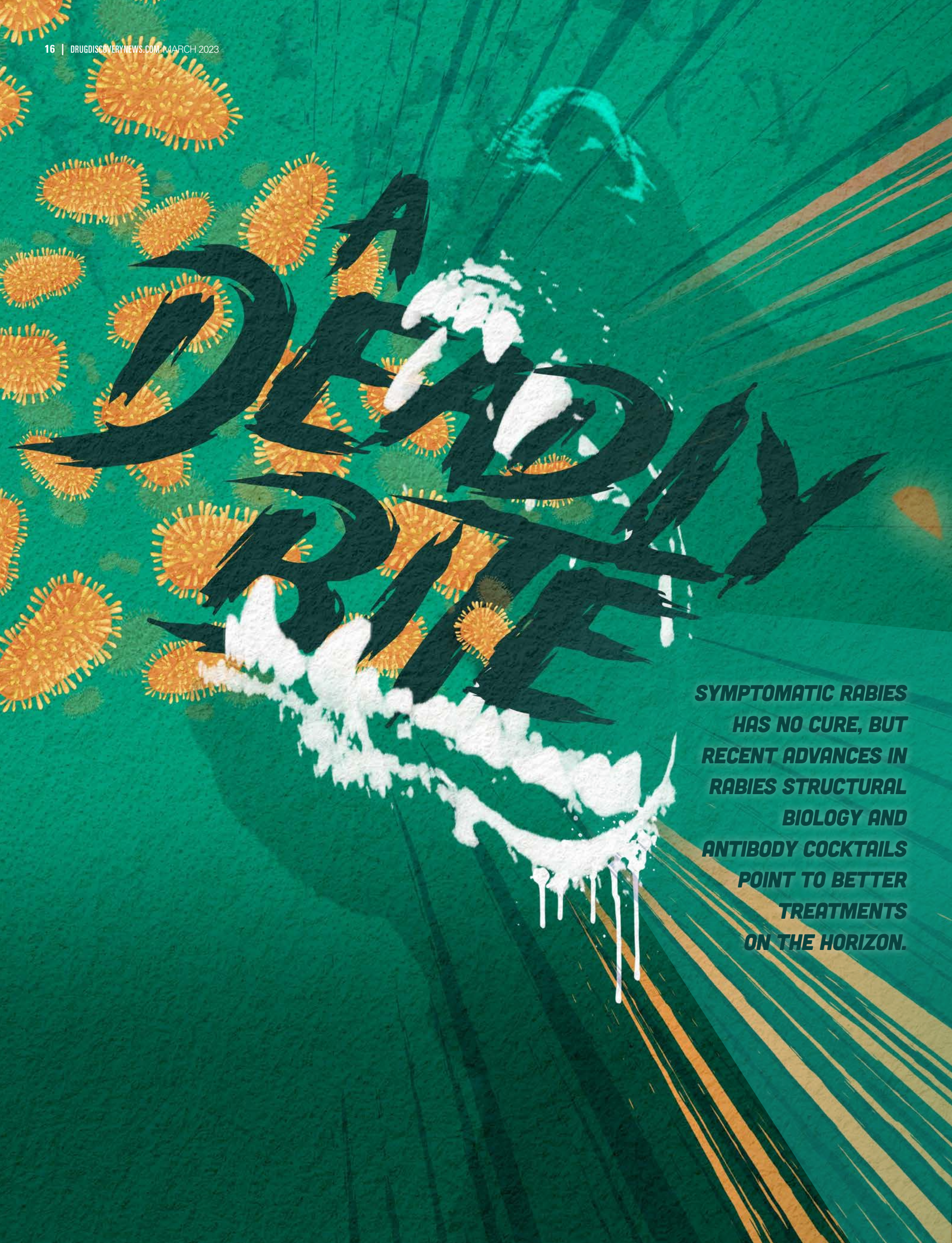

DEAD RISE

SYMPTOMATIC RABIES HAS NO CURE, BUT RECENT ADVANCES IN RABIES STRUCTURAL BIOLOGY AND ANTIBODY COCKTAILS POINT TO BETTER TREATMENTS ON THE HORIZON.

BY STEPHANIE DEMARCO, PHD

THE ANCIENT MESOPOTAMIANS may not have known what caused their dogs to lash out and seize, but they knew that if one of these dogs bit a human, the results would be deadly. The first written record of rabies dates back to 1930 BCE in the Mesopotamian Laws of Eshnunna, but it likely existed long before that (1).

"We've known about it as long as there have been human stories," said Erica Ollmann Saphire, a structural biologist and infectious disease researcher at the La Jolla Institute for Immunology.

While the Mesopotamians realized that rabies came from a dog's saliva, it took another 3,700 years to prove it. In 1804, German physician Georg Gottfried Zinke showed that injecting saliva from a rabid dog transferred the infection to a healthy dog (2). Louis Pasteur and his colleagues at the École Normale Supérieure found that if they transferred the saliva from a rabid dog to a monkey and then from that monkey to another one, the virus weakened with each new transfer. Using that attenuated rabies virus, Pasteur developed the very first vaccine for rabies in 1885 (2).

Nowadays, there is an inactivated rabies virus vaccine for people at high risk for contracting rabies. But while effective, the vaccine does not give long lasting immunity. People living at risk for contracting rabies need to get regular booster shots every three years or so (3).

If people who have not been vaccinated get bitten by a suspected rabid animal, postexposure prophylaxis (PEP) is an effective treatment if taken soon after the bite. PEP consists of both the rabies vaccine and human rabies immunoglobulins to neutralize the virus before the vaccine takes effect. But once rabies symptoms begin, the disease is practically 100 percent fatal. At that point in the infection, there is no treatment.

In wealthy nations like the United States, rabies cases from dog bites are rare, but about 50,000 people require PEP treatment for encounters with bats, skunks, raccoons, and other wild animals (4). The Centers for Disease Control and Prevention (CDC) estimates that the US spends anywhere from $245 to $510 million every year on rabies prevention and treatment. In countries with fewer resources, the outlook is bleaker. Many cannot afford PEP.

"The rabies immunoglobulin, which is a component of PEP, is also the most expensive part because it's made from human or equine" immunoglobulins, said Todd Smith, an infectious disease researcher at the CDC. "A lot of times they'll receive either the equine product… or they will just receive vaccine with no passive antibody to protect."

With more than 3 billion people living at risk for rabies infections worldwide and approximately 50,000 to 60,000 people dying from the infection every year, better and more accessible preventative care and treatments are still needed (5).

"In the Kenyan setting where I work, we've had five children this year — in fact, within the last six months — come through with rabies," said Darryn Knobel, a rabies researcher at Ross University.

Stymied by a lack of funding and interest, new rabies treatments and improved vaccines have stagnated. But recent achievements in mapping the full structure of the rabies glycoprotein and advances in neutralizing monoclonal antibody cocktails are bringing potential new rabies therapies and vaccines to the forefront, giving hope for making rabies a completely treatable infection.

> "WE'VE KNOWN ABOUT IT AS LONG AS THERE HAVE BEEN HUMAN STORIES."
>
> *– Erica Ollmann Saphire, La Jolla Institute for Immunology*

A sneaky killer

Despite being a tiny virus with only five proteins at its disposal, rabies does a lot of damage. The rabies virus is a member of the lyssavirus genus — lyssa meaning "rage" or "fury" in Greek — a group of bullet-shaped viruses that preferentially infect neurons.

When an animal infected with rabies bites a human or another animal, the rabies virus typically enters the host's neurons at a neuromuscular junction at the bite site. Rabies only expresses one protein on its surface: its glycoprotein. With that single protein, it binds to a number of different cellular receptors to make its way into neurons.

This "is one of the reasons why it's so hard to combat and develop a good therapy because it has lots of ways of getting into a cell," said Maegan Weltzin, an ion channel physiologist at the University of Alaska Fairbanks who studies the interaction between rabies virus and nicotinic receptors.

Once inside a neuron, the virus travels through the host's peripheral neurons, making its way to the brain (6). For this reason, rabies bites to the face are more serious than bites to the legs. The neurons in the face are much closer to the brain, so the virus can get to the brain faster. While rabies travels through the peripheral nerves, infected patients may have some mild flu-like symptoms and pain at the bite site. At this point, PEP treatment is likely still effective.

Surprisingly, the immune system doesn't mount a strong response to the virus while it's in the peripheral nervous system. Some researchers hypothesize that this may be because the rabies virus glycoprotein is structurally heterogeneous, perhaps making it difficult for the immune system to generate effective neutralizing antibodies.

Once the virus reaches the brain, all bets are off. As it begins to replicate in the brain, it causes neuronal dysfunction, and the classical clinical rabies symptoms emerge: agitation, anxiety, hyperactivity, difficulty swallowing, fear associated with swallowing water, and hallucinations among others.

Once patients reach this stage, the only therapeutic option is to keep them comfortable. There have been some anecdotal reports of patients recovering from this kind of clinical rabies infection by being placed in a coma and treated with the rabies vaccine, rabies immunoglobins, antivirals, and ketamine (7). But with such a high failure rate, many clinicians recommend against this treatment avenue.

Although the virus wreaks havoc inside the brain, the overall brain structure looks normal.

"The virus really needs intact neuronal architecture in order to spread trans-synaptically, so it's going out of its way, at least from an evolutionary perspective, to avoid triggering that immune system that could actually cause that kind of damage," said Knobel. "It's one of the reasons why I have some optimism for a treatment because, yes, definitely some degeneration or dysfunction has been triggered. But by and large, the architecture remains intact."

Knobel and his team hypothesize that a rabies viral infection is essentially "an extremely acute, rapidly progressive neurodegenerative disease," he said. Using that idea as a starting point, he and his collaborators are looking for neurodegenerative disease biomarkers in dog rabies patients to see if they can shed light on rabies disease processes. They have begun examining infected neurons *in vitro* and hope to advance their studies into animal models in the future.

"Really getting to understand what is going on in those neurons, I think is a key area of this translational kind of research," said Knobel.

Silver bullet antivirals and monoclonals

A good rabies treatment will catch the virus before it reaches the brain. Researchers are exploring antiviral and monoclonal antibody therapies. So far, there are no antivirals that work against rabies, but that hasn't stopped Smith and his team at the CDC from looking.

"We routinely screen antiviral compounds and drugs that we hear about or that we're working on for other viruses," said Smith. Recently, he and his team came across a proprietary form of the antiviral ranpirnase called TMR-001 (8). The antiviral seemed to be effective against poxviruses, so Smith and his team decided to see how it fared against rabies virus.

"It worked really well in cell culture, but it did not protect animals, which is not uncommon for rabies antivirals," said Smith. "Part of that is just the problem of drug delivery. Getting the drug to the right place at the right time and having it be active enough to slow down and stop the virus… is really difficult once that cascade in the central nervous system starts."

Smith and his team haven't given up hope for an antiviral strategy. They are looking for other antivirals to test against rabies and for approved drugs that may also have antirabies effects.

"That's the silver bullet that everybody in the rabies field is always looking for — the treatment piece of it," said Smith. For now, he and his team have shifted their focus to improving PEP with monoclonal antibodies. The human rabies immunoglobulins in PEP consist of a mixture of antibodies that a person's immune system produces in response to rabies vaccination.

"Monoclonal antibodies have been proposed a long time ago, probably 30 years ago, as a potential candidate to replace the rabies immunoglobulin," said Smith. "The idea is that a cocktail of monoclonal antibodies could be produced on a larger scale and cheaper than the current immunoglobulin product," which requires human donors.

So far, the only approved monoclonal antibody product for rabies is in India where the main source of rabies infections is dogs. In the United States, where rabies infections typically come from bats, a single monoclonal antibody likely would not provide sufficient treatment because bats typically carry many different rabies virus variants. Instead, a monoclonal antibody cocktail could work.

Smith and his team recently collaborated with researchers at MassBiologics of the University of Massachusetts Chan Medical School to identify a monoclonal antibody cocktail against the main strains of rabies virus found in North America (9). The first antibody cocktail they tested had some trouble neutralizing some of the strains, but when they switched to their second mix, it neutralized all of the rabies variants tested.

"That's what we always hope," Smith said. Moving forward, Smith and his team have additional ongoing collaborations testing other monoclonal antibody cocktails against these rabies variants.

A glycoprotein breakthrough

The biggest hurdle facing the development of better rabies vaccines and therapies has been that no one really knows what the rabies surface glycoprotein looks like.

"Even when I was a graduate student in the early 90s, people were trying to get the structure of the surface protein of rabies because of its fundamental importance to human health and to animal health, and it hadn't yet been possible," said Saphire. "It's shocking, given how fundamentally important rabies is to human and animal health, that we know so little about how it interacts with its receptors and what the human immune response might be."

Because of the heterogeneity and flexibility of the rabies glycoprotein, prior attempts to determine its structure required researchers to modify the protein to crystalize it. In a recent attempt, researchers mapped the monomer of the glycoprotein, but they had to chop off flexible pieces of the glycoprotein called the fusion loops to map it, meaning that a lot of structural information about the trimeric protein was still missing (10).

"Sometimes the most potent antibodies are those that bridge different pieces of the glycoprotein together. They bridge the subunits, or they bridge the different monomers and the trimer," said Saphire. "To evaluate what those most potent and most protective kinds of antibodies might be, we needed to understand exactly how all the different domains and different monomers get gathered together in that final structure."

The key hurdle that Saphire and Heather Calloway, the postdoctoral fellow in her laboratory who led the project, needed to surmount was how to stabilize the glycoprotein's flexible fusion loops in their natural conformations.

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First, Callaway and her colleagues bound the glycoprotein with a neutralizing antibody, which locked the rabies glycoprotein in its prefusion form — the form it takes before it infects a cell. With the glycoprotein locked in place, Callaway isolated it in a detergent to keep the protein in its native state. She and her team then took advantage of cryo-electron microscopy's (cryoEM) unique capability to capture flexible and heterogeneous structures to solve the glycoprotein's prefusion structure (11).

"You see within the cryoEM that it does have multiple conformations, so if you put them all together, you can see sort of a motion. It's almost like the fusion loops are dancing," said Callaway.

Unlike other viral glycoproteins, which typically keep their fusion loops tucked on the inside of the proteins, the rabies virus glycoprotein's fusion loops gather at its base to help stabilize the protein in its trimeric form. "This explains why that structure had been so elusive for so long," Saphire added.

After imaging at least 100,000 rabies virus glycoprotein particles over three years, Callaway, Saphire, and their team finally revealed the roadmap to better rabies vaccines and antibody therapies. With a clear and complete structure, they and other researchers can study where other neutralizing antibodies bind and engineer antibodies that bind the rabies prefusion glycoprotein better.

Despite infecting humans for thousands of years, rabies infections that reach the brain still have no cure.

When Louis Pasteur developed a vaccine for rabies in 1885, it was only the second vaccine ever invented.

Dogs infected with rabies transmit the virus via their saliva, typically through a bite.

With increasing contact between humans and bats, scientists worry that rabies-related viruses that cause the same symptoms as rabies but don't respond to rabies treatments will jump into humans.

Saphire and her team are now studying the structure of the rabies glycoprotein when bound to different antibodies. They're also designing more precisely engineered vaccines for other lyssaviruses that look like rabies but do not respond to rabies vaccines or PEP treatments such as the Australian bat lyssavirus (ABLV) and European bat lyssavirus (EBLV), which cause symptoms identical to rabies in humans (12).

"Many of them exist in bats and can infect humans," said Callaway. "Have they gotten out of bats and into other carnivore populations like rabies? Not yet, but they could. And if they did, we would like to have vaccines or antiviral antibodies already ready to combat this kind of potential pandemic."

With increased human encroachment on bat ecosystems, the potential for bat and human interactions increases. More rabies researchers such as Matthias Schnell at Thomas Jefferson University are developing vaccines against rabies-related viruses. He uses a deactivated rabies virus as a vaccine platform, and he and his team are creating new vaccines based on the antigenic regions of Mokola lyssavirus and Lagos bat lyssavirus.

"It's really hard to start from zero," said Schnell. "You really don't know when you will need it."

Daring small molecules

With new antivirals, monoclonal antibodies, and vaccines all in the works, researchers at the drug discovery company Prosetta Biosciences take a unique approach to rabies therapy: small molecules.

Like all viruses, rabies virus hijacks host proteins to replicate. Vishwanath Lingappa, a molecular biologist and leader of Prosetta Biosciences, realized that instead of targeting the virus, they could make an antiviral to take back the hijacked host proteins.

In earlier research, Lingappa's colleagues discovered that host proteins form transient multiprotein complexes to help assemble viral capsids (13). Lingappa and his colleagues reasoned that if they found a small molecule that impaired the host multiprotein complex that the viruses use to replicate and assemble, they could interfere with viral assembly. But their path to searching for rabies antivirals was as unconventional as their approach.

"We did it actually almost on a dare," said Lingappa. As he explained his small molecule antiviral approach to a virologist colleague, "he said, 'no, there are no small molecules against rabies.' I said, 'Well, we can find small molecules for any viral family.' He wouldn't believe it, and I said, 'Okay, you've pissed me off, so I'm going to find them.'"

To find small molecules that bound and impaired host proteins that aid rabies virus assembly, Lingappa took advantage of his expertise in cell-free translation. Using Prosetta Biosciences' library of small molecules, he and his team screened for molecules that interfered with rabies virus capsid formation in a cell-free translation assay (14). They found that a molecule called PAV-866 targeted a portion of the host cell's supply of the protein ABCE1 and blocked rabies virus assembly *in vitro*.

"Of all the ABCE1 in the cell, only about a few percent are in the multiprotein complex that rabies needs. The other 95 plus percent are doing other things," said Lingappa. Because of that, an approach like CRISPR or siRNA knockdown won't solve the problem of only inhibiting the subpopulation of the protein that the virus needs.

In their recent work, Lingappa and his team figured out that to co-opt host machinery, viruses bind to an allosteric site on the target protein to help form a capsid. By binding to the protein's allosteric site, "the virus tweaks it this way. Our small molecule tweaks it that way," impeding viral assembly in the process, said Lingappa. Their most advanced candidate based on this mechanism is a SARS-CoV-2 small molecule antiviral (15).

Since their *in vitro* success with PAV-866 and other small molecules that inhibit rabies virus assembly, the team has pushed these candidates even further.

"We sent them to the CDC. They said, 'oh my gosh, these work,'" said Lingappa. That finding "was our first identifying of a small molecule that was efficacious in cell culture. We've subsequently advanced that. I have a molecule that shows efficacy in mice infected with rabies, even when you wait until the animals have rabies in the brain," he added. "It wasn't complete efficacy, but it was certainly a great start."

Lingappa plans to begin studies for this rabies therapeutic and to one day get them into human clinical trials, but like many researchers working on rabies, he hopes to first find funding to get the studies up and running.

"We've almost convinced ourselves that rabies is untreatable and always will be," said Knobel. "But I think it's really time to revisit that and really try and imagine rabies as a treatable disease and do the kind of research and development that will bring us there." ■

CREDIT: VISHWANATH LINGAPPA

Vishwanath Lingappa develops small molecules to treat viral diseases such as rabies.

CREDIT: LA JOLLA INSTITUTE

Heather Callaway led the team that solved the complete structure of the rabies surface glycoprotein.

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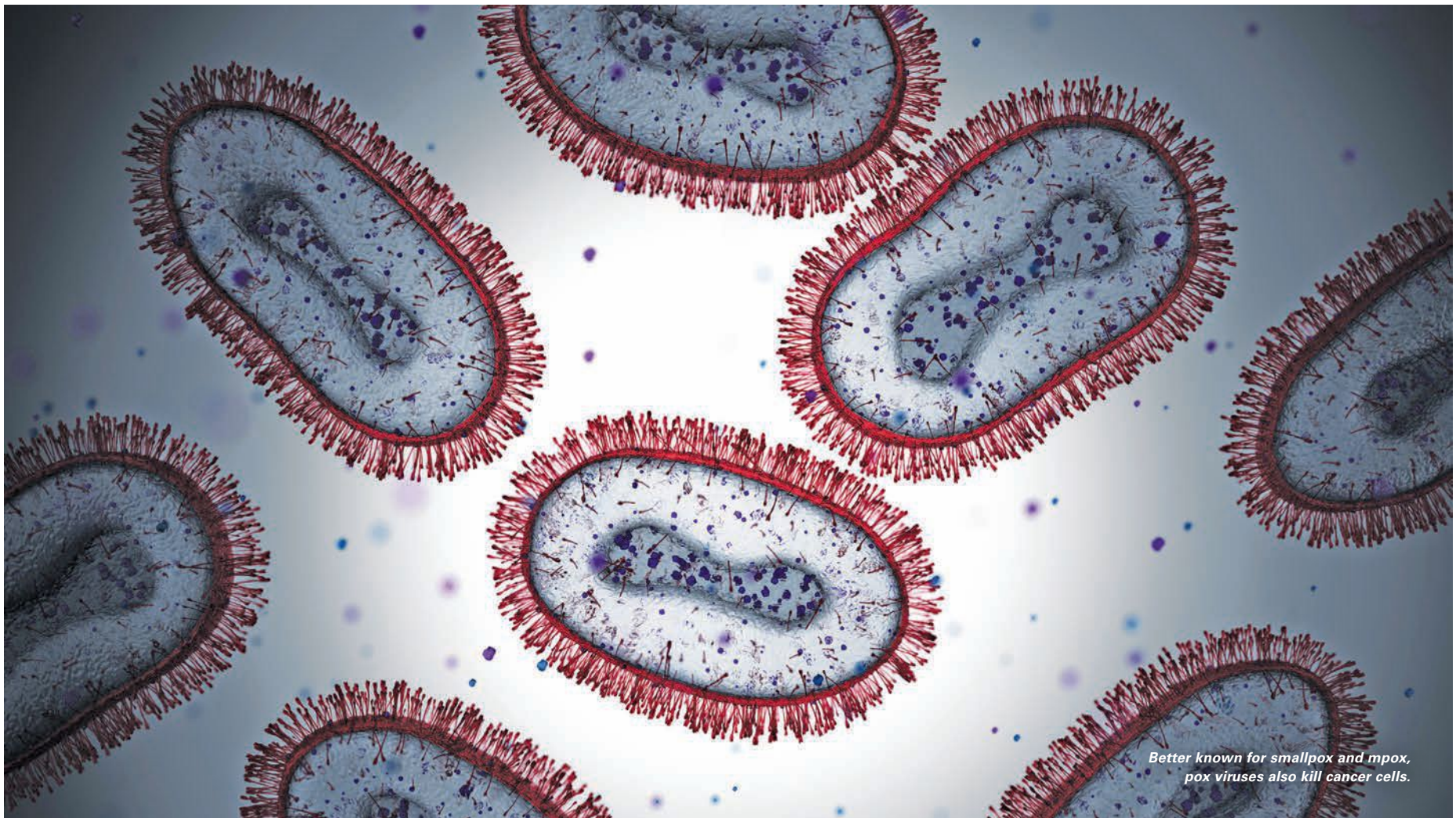
cell therapy

A Pox on Cancer

The live virus in the smallpox vaccine boosts the activity of cancer-killing immune cells for hard-to-treat tumors.



Better known for smallpox and mpox, pox viruses also kill cancer cells.

BY STEPHANIE DEMARCO, PHD

While pox viruses call to mind images of pus-filled blisters and painful sores, a few clever genetic tweaks turn these viruses from deadly villains into cancer-fighting heroes.

Best known as the live virus present in the smallpox vaccine, vaccinia virus is surprisingly good at killing cancer cells. With a natural affinity for tumors, a fast replication time, and a good safety profile, pox viruses have the potential to treat deadly and treatment-resistant cancers, including pancreatic and brain cancers.

"We're presenting a virus that basically saved the world from the scourge of smallpox," said Nicholas Lemoine, a cancer researcher at VacV Biotherapeutics and Queen Mary University of London who is developing a vaccinia virus-based cancer treatment. "What actually came from vaccination has shown us that this type of platform is something we should never forget about."

Cancer fighting viruses work by infecting tumor cells and doing what viruses do best: making more of themselves. After co-opting the tumor's cellular machinery to replicate their genetic material, the viruses burst out of the cell, leaving an explosion of dead cancer cell debris in their wake. Patrolling antigen presenting cells of the immune system pick up these bits of dead cancer cells and use them to prime other immune cells like T cells to recognize and destroy cancerous cells.

"The cancer cells themselves are basically giving up their arms and presenting them to the invading army of the adaptive immune response," Lemoine said. By stimulating the host's immune system to recognize tumors, these viruses make it possible for T cells to find and kill tumors that may have spread to other parts of the body.

Scientists recognized the potential anticancer effects of viruses when they discovered the first oncolytic virus in 1992. When they injected a genetically modified herpes simplex virus into a brain tumor in a rat, the tumors shrank significantly (1). Since then, researchers have tested a variety of different cancer fighting viruses in preclinical and clinical trials, and regulators have approved four for use in humans so far. Regulators in Latvia approved an unmodified picornavirus to treat melanoma in 2004, and the National Medical Products Administration approved adenovirus H101 to treat head and neck cancer in China in 2005 (2). Other regulatory bodies, however, have not approved these treatments in other places yet. In the United States, Europe, and Canada, regulators approved a modified herpes simplex virus called T-VEC (brand name Imlygic) to treat certain melanomas in 2015. In 2021, Japanese regulators gave limited approval to another modified herpes simplex virus named Teserpaturev/G47Δ (brand name Delytact) for brain cancer treatment.

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>
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Although approved, the main challenge with these virus-based cancer treatments is that they're not always effective. Recent research on vaccinia virus in combination with other drugs suggests that the pox virus may be a safe and more effective solution.

Due to its use in the smallpox eradication campaign in the 1960s and 70s, the world already considers vaccinia virus quite safe. But the virus also has multiple inherent features that make it particularly adept at targeting cancer.

For one, vaccinia virus has a natural preference for infecting cancer cells over normal ones, which scientists noticed when fluorescently labeled virus accumulated in a mouse brain tumor but not in healthy cells (3). Unlike many other viruses, vaccinia virus doesn't need

to bind to a specific cellular receptor to enter a host cell; it infects them via endocytosis (4). Vaccinia virus also thrives in low oxygen conditions often associated with solid tumors (5). Once inside the cell, vaccinia virus replicates in the cytoplasm, avoiding any interaction with host cell DNA in the nucleus. This behavior led many scientists to consider the pox virus safer than therapies based on herpes virus and adenovirus, which replicate in the nucleus.

Researchers wanted to improve vaccinia virus's tumor specificity even more. While the virus encodes a number of different genes to help it replicate inside host cells, "people discovered that those genes are kind of redundant for the virus to infect and replicate in cancer cells," said Zong Sheng Guo, a cancer researcher at Roswell Park Comprehensive Cancer Center.

One of these viral genes encodes for the enzyme thymidine kinase, which catalyzes a critical step in the synthesis of thymidine triphosphate. Because cancerous cells rapidly divide, they have a large pool of thymidine nucleotides for the virus to use. To make vaccinia virus more selective for cancer cells, scientists deleted its thymidine kinase gene, ensuring that the virus can only replicate inside and lyse cancer cells but not healthy cells, which divide less frequently and therefore don't maintain a pool of thymidine.

These advantages propelled vaccinia virus-based therapies into multiple clinical trials in recent years, but like their other oncolytic viral counterparts, their potency has been low. One reason for this may be that alone, vaccinia virus lysis of cancer cells may not stimulate the host immune system as well as it needs to. Researchers wondered if pairing vaccinia viral therapy with immune checkpoint inhibitors or other immune modulating treatments might amplify its power.

"I have been interested in combination [therapies] with small molecules targeting different key signal pathways," said Guo. Using a vaccinia virus that he and his colleagues developed, Guo and his team administered the virus with a small molecule activator of ferroptosis, an iron-dependent cell death pathway, to mouse models of hepatocellular carcinoma and colon cancer (6-7). The combination treatment shrank the tumors more than either treatment alone.

CREDIT: VACV BIOTHERAPEUTICS

Nicholas Lemoine and his colleague Yaohe Wang develop cancer fighting pox viruses.

"If we can use this platform to save a huge number of people from bad outcomes with cancer using a virus that basically saved the world from smallpox, I think that's a good story to tell."

– Nicholas Lemoine,
VacV Biotherapeutics and Queen Mary University of London

"It's a great result. We like it. We just need to know more [about] how they work together, and in the future, we can improve our strategy to enhance the efficacy even further," said Guo.

The best studied vaccinia viral treatment is JX-594 (brand name Pexa-Vec), a vaccinia virus expressing the immune-activating gene granulocyte-macrophage colony-stimulating factor (GM-CSF) and an inactivated thymidine kinase gene.

Researchers at the National Institutes of Health (NIH) led by cancer researcher Tim Greten recently presented data from a phase 1/2 study using Pexa-Vec in combination with one of two different immune checkpoint inhibitors in patients with refractory colorectal cancer (8). Only some types of colorectal cancer respond to immune checkpoint inhibitors, but Greten hoped that combining the treatments with the oncolytic vaccinia virus would improve outcomes for patients with advanced colorectal cancer.

"I was hoping for a stronger response," Greten said. "We had a patient who showed a significant response and a long-lasting response, and you ask, why is it one and not the others?"

Despite the lackluster results, Greten and his team still believe in the oncolytic virus strategy to help treat these difficult cancers. "They work," he said. "There's a lot of data suggesting that this type of treatment actually does make sense." He and his team plan to keep testing different combinations of the virus and immune modulating drugs to find the most effective treatment for cancer patients.

Although not as far along as Pexa-Vec, another vaccinia virus strategy developed by Lemoine and his colleague Yaohe Wang at VacV Biotherapeutics recently demonstrated positive results in mouse and hamster cancer models.

Wang's team recently discovered a way to increase the amount of vaccinia virus that infects tumors. Vaccinia virus can form extracellular enveloped virions (EEVs) where the virus hides inside an envelope made up of the tumor cell's surface membrane, disguising the virus from the immune system. The researchers noticed that when they delivered the vaccinia virus treatment intravenously, host macrophages cleared most of the virus before it arrived at the tumor (9). To take up the vaccinia virus, macrophages depended on an enzyme called PI3 kinase (PI3K) delta. When the team intravenously administered both their vaccinia virus and a clinically licensed PI3K inhibitor, they saw increased vaccinia virus at the tumor and tumor growth slowed in mouse models of colorectal or breast cancer, increasing their survival.

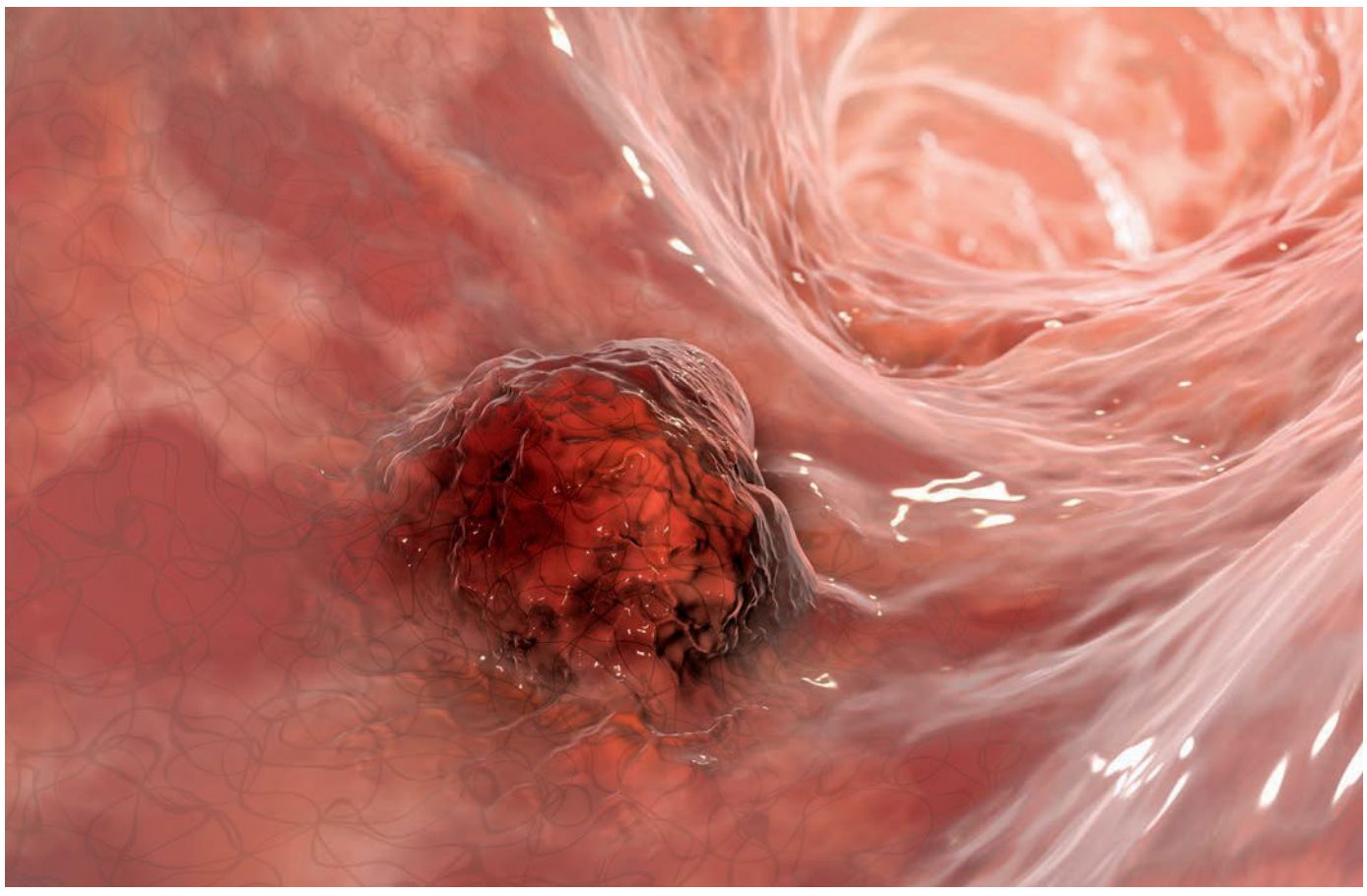
"The PI3 kinase delta inhibitor is essentially rolling the pitch in order to suppress macrophages, which would otherwise engulf the viruses that you then deliver," said Lemoine.

Wang and Lemoine recently tested their vaccinia virus expressing the cytokine interleukin-21 in mice with glioblastoma (10). When they injected the mice intravenously with this engineered virus and also gave mice the immune checkpoint inhibitor α-PD1, the treatment slowed tumor growth and, in some cases, eliminated tumors altogether. The mice also survived longer.

The team also slowed pancreatic tumor growth with their engineered vaccinia virus combined with α-PD1 in mice and hamsters (11). In particular, the researchers found that when used together, the vaccinia virus sensitized tumors that were previously resistant to α-PD1 treatment by priming the immune system.

"When we found positive results in tumor systems that had basically failed to respond to anything else, we redoubled our efforts to bring this to clinic," said Lemoine. The team plans to begin clinical trials sometime in mid-2024.

"If we can use this platform to save a huge number of people from bad outcomes with cancer using a virus that basically saved the world from smallpox, I think that's a good story to tell," said Lemoine. ■



Researchers hope that an engineered vaccinia virus can target difficult-to-treat cancers such as colorectal cancer, brain cancer, and pancreatic cancer.

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A Synthetic Switch for Safer CAR T Cell Therapy

By activating CAR T cells only in the presence of an antibody switch, Travis Young and his team hope to make this cancer treatment safer and more effective.

Switchable CAR T cells only attack tumor cells in the presence of an antibody switch.

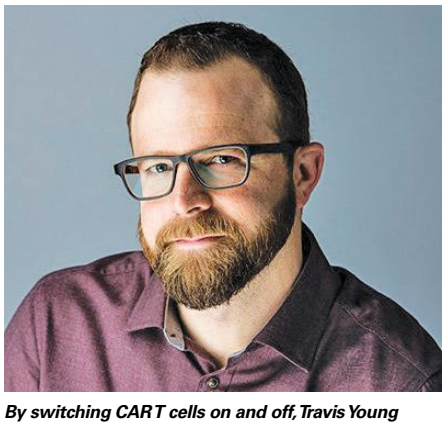
INTERVIEWED BY STEPHANIE DEMARCO, PHD

LIKE MINIATURE TERMINATORS, chimeric antigen receptor (CAR) T cells seek out and destroy cancer cells autonomously. But sometimes these cancer targeting assassins — which scientists engineer to recognize specific cancer cell markers — get a little out of control.

"We've got CAR T cells expanding exponentially," said Travis Young, a molecular biologist who leads the Biologics division of Calibr, the translational research arm of Scripps Research Institute. "If they expand too rapidly and during that expansion, release a lot of cytokines and have a major inflammatory response, then you end up with cytokine release syndrome (CRS)… or neurotoxicity."

While clinicians can treat patients who develop CRS or immune effector cell-associated neurotoxicity syndrome (ICANS), severe cases can be fatal (1). With his background in synthetic biology and protein engineering, Young and his team developed a strategy to prevent CAR T cell runaway expansion: switchable CAR T cells.

From his start helping his college roommates with their chemistry homework to figuring out how to design a clinical trial with brand new technology, Young hopes to make CAR T cell therapy safer for patients. With six out of nine lymphoma patients



CREDIT: DON BOOMER

By switching CAR T cells on and off, Travis Young hopes to prevent dangerous side effects associated with CAR T cell therapy.

"In my second year, my roommates were all doing the New York Times crossword puzzle, and I was doing their organic chemistry homework."

– Travis Young, Scripps Research Institute

already showing a complete response to his CAR T cell therapy in an ongoing phase 1 clinical trial, Young and his colleagues are just getting started with demonstrating what switchable CAR T cells can do.

How did you first become interested in science?

One year when I was a kid, my grandfather, who worked at Brookhaven National Laboratory on Long Island where I grew up, got me a chemistry set for Christmas. But I never really picked up chemistry until I got to college. I enrolled as a computer science major because I thought that was going to be where all the jobs were. In my second year, my roommates were all doing the New York Times crossword puzzle, and I was doing their organic chemistry homework. I realized then that I had a knack for the sciences. I could probably do chemistry better than I could read and write. I changed my major to biochemistry, and I really enjoyed it.

How did you end up working on CAR T cell therapy?

I earned my PhD at Scripps Research Institute with Peter Schultz where I worked on a platform called unnatural amino acid mutagenesis. It allowed us to use a 21st amino acid in the genetic code to endow proteins with unique chemistries and functions. From there, I was a postdoctoral researcher in Christopher Walsh's lab at Harvard Medical School in natural products. I became a gene jockey there. I became very proficient at extracting genes and cloning them in different cassettes. When I started my lab at Calibr, I had a lot of experience in molecular biology, synthetic biology, and protein engineering that lent itself to developing our switchable CAR T cell platform technology.

Why create switchable CAR T cells?

CAR T cells are remarkable. Endowing a cell with a gene that allows it to seek and destroy tumors and allowing the patient cells themselves to be the drug is a very powerful approach. Because it's a completely different mechanism from traditional chemotherapies, it is effective against chemotherapy-resistant hematological cancers.

That difference leads to new problems, however. When we genetically engineer a CAR T cell and transfer it back into a patient, it is autonomous. The CAR T cell expands when it finds tumor cells so that it can find and lyse more tumor cells. That expansion of the drug in the body is the opposite of the central dogma of drug development to date, which is that when we give a subject an antibody or small molecule drug, there's a decay over time until the drug is eliminated. That presents challenges because if we don't know what the end exposure of our cells is going to be, we can end up with CRS or ICANS.

When we looked at this problem, we wanted to come up with a simple solution to leverage the potency of these CAR T cells and their abilities to seek out and destroy tumor cells, but we wanted to restore the traditional pharmacological control that we get from dosing a typical antibody or small molecule drug. That was the inception of the switchable CAR T cell program. The very basic, fundamental idea was to regain control of the cells.

How do switchable CAR T cells work?

Instead of binding to cancer cells, the CAR T cells turn on when they bind to an antibody that we create, which we call a switch. We target the antibody switches to cancer antigens such as CD19, CD20, or CD22. We deliver the engineered CAR T cells to the patient, and then when we deliver the switch, it turns the CAR T cells on when the switch sees a tumor. When that antibody switch is naturally eliminated and no longer present in the body, then the CAR T cells turn off, just like a normal drug.

We think of it like a software-hardware based approach because we're giving patients these genetically engineered cells — the hardware — but the cells don't do anything without software, which is the antibody switch. The response is easily tuned by the amount of the antibody switch that we give the patient. Now we've got all of these different levers for how we can control these CAR T cells in the body.

CREDIT: FILIP TRPCHEVSKI

Travis Young and his team developed switchable CAR T cells to make cancer treatment safer and more effective than treatments with standard CAR T cells.

> "We think of it like a software-hardware based approach because we're giving patients these genetically engineered cells — the hardware — but the cells don't do anything without software, which is the antibody switch."
>
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Have you noticed fewer CRS and ICANS events with switchable CAR T cells?

Yes, and not only can we minimize the duration of adverse events such as CRS and ICANS, but when we stop dosing the switch, the CAR T cells can rest. One of the major challenges in typical CAR T cell therapy is preventing T cells from getting exhausted. We've demonstrated in our preclinical models that this rest phase when there's no switch present actually makes the CAR T cells more potent and more durable over time. We're on a path to demonstrate that in the clinic as well.

We're still learning what we can do with these switchable CAR T cells and the different ways we can modulate their responses. The thing that excites me from a basic science perspective is that this is a much more physiological way of activating a T cell. A T cell responds when there's an infection by activating, expanding, eliminating the infection, and then contracting. They then enrich a central memory CAR T cell population, which is ready to protect against subsequent infections. That's the way that this switchable platform is intended to work, except that the antibody switch now causes the activation, the rest, and the reactivation.

What was the biggest challenge you faced in setting up your clinical trial for switchable CAR T cells?

Going into it, there was a lot that we didn't know! The immune systems of the preclinical models that we tested didn't fully recapitulate the human immune system, so it was going to be difficult to determine how to dose the CAR T cells and the antibody switch. Neither the engineered CAR T cells nor the antibody switch had been in a patient before, so we wanted to start the dose at a level that was safe. But at the same time, we didn't want to start exceedingly low because we didn't want to waste a lot of patients' time with potentially subefficacious doses. Because of the complexity and time that it takes for these people to receive therapy, we wanted to make sure that everybody had a shot at a response.

When we looked at the data from the first three subjects, two out of the three had complete responses to the therapy with the lowest doses of our antibody switch and the lowest dose of our cells. Compared to a lot of other antibody-based drugs, which are dosed at 10 milligrams per kilogram or higher, we dosed ours at just 10 micrograms per kilogram. It's 1000-fold lower than those other drugs, so it's a very potent system.

What was your reaction to seeing those first complete responders?

When we saw that two out of the first three subjects responded, it was really gratifying. The effort from the team over the last ten years resulted in something that was, first and foremost, safe. Second, from there on out, we knew that we were going to provide every subject with the opportunity to respond to the therapy.

Are you following the same patients to monitor the durability of the switchable CAR T cell treatment?

We are. The tests are still in their early stages, but it looks promising with regards to durability. When we reported the first set of data in September 2022, we announced that the majority of the subjects had complete responses after just one or two cycles of the antibody switch. In the trial, the patients received up to six cycles, and as we administered more cycles, we saw a deepening of their response to the therapy. We saw further decreases in lesion sizes, and during that period, we didn't see any tumor relapses. One person had a tumor come back about a year later, but the other responses were still maintained during this period. We're continuing to follow those patients, and we're optimistic about the treatment durability.

Could switchable CAR T cells also treat solid tumors?

Solid tumors are the next frontier for CAR T cells. Folks have put forward many different programs using CAR T cells to target solid tumors, but there are some challenges in solid tumors that are not shared with hematological cancers. The first is the choice of the antigen. When we engineer a CAR T cell for a B cell malignancy, the most common antigen that we choose is CD19 because it's expressed on those tumor cells. But normal healthy B cells also express CD19. So, when a subject receives CAR T cells targeting CD19, the collateral damage is healthy B cells, but people can live fairly normal and healthy lives with depleted B cells.

In solid tumors, the antigens are also present on vital tissues that we can't do without. For example, in breast cancer, HER2 is a common tumor antigen to target, but it's also expressed on lung tissues and in some places in the heart. Collateral damage there is not tolerable. There are no truly unique solid tumor antigens that lack expression in vital tissues. That's why creating a CAR T cell for a solid tumor is so difficult.

Because we can tune the level of activity of our CAR T cells with the switchable platform, we may find a therapeutic index where we can target HER2 on a breast cancer cell but not in healthy tissue. We think that pharmacological control is going to be a major advantage in using CAR T cells for solid tumors. We're working in collaboration with AbbVie applying what we've learned now with switchable CAR T cells in liquid tumors to solid tumors, and we're very excited about that partnership.

What has been the most rewarding aspect of working on this project so far?

Every time we have a tumor scan come back from a patient that still shows a complete response, that is the most rewarding thing. To completely eliminate tumors in preclinical models is exciting, but there are lots of things in preclinical models that cure cancer. Trying to recapitulate those findings in a patient is always the challenge. To see that the fundamental science behind this bore out in patients is really gratifying to me. ■

This interview has been edited for length and clarity.

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explained

HOW DOES CAR T CELL THERAPY WORK?

By engineering immune cells to effectively attack cancer cells, researchers established a new paradigm for cancer therapy.

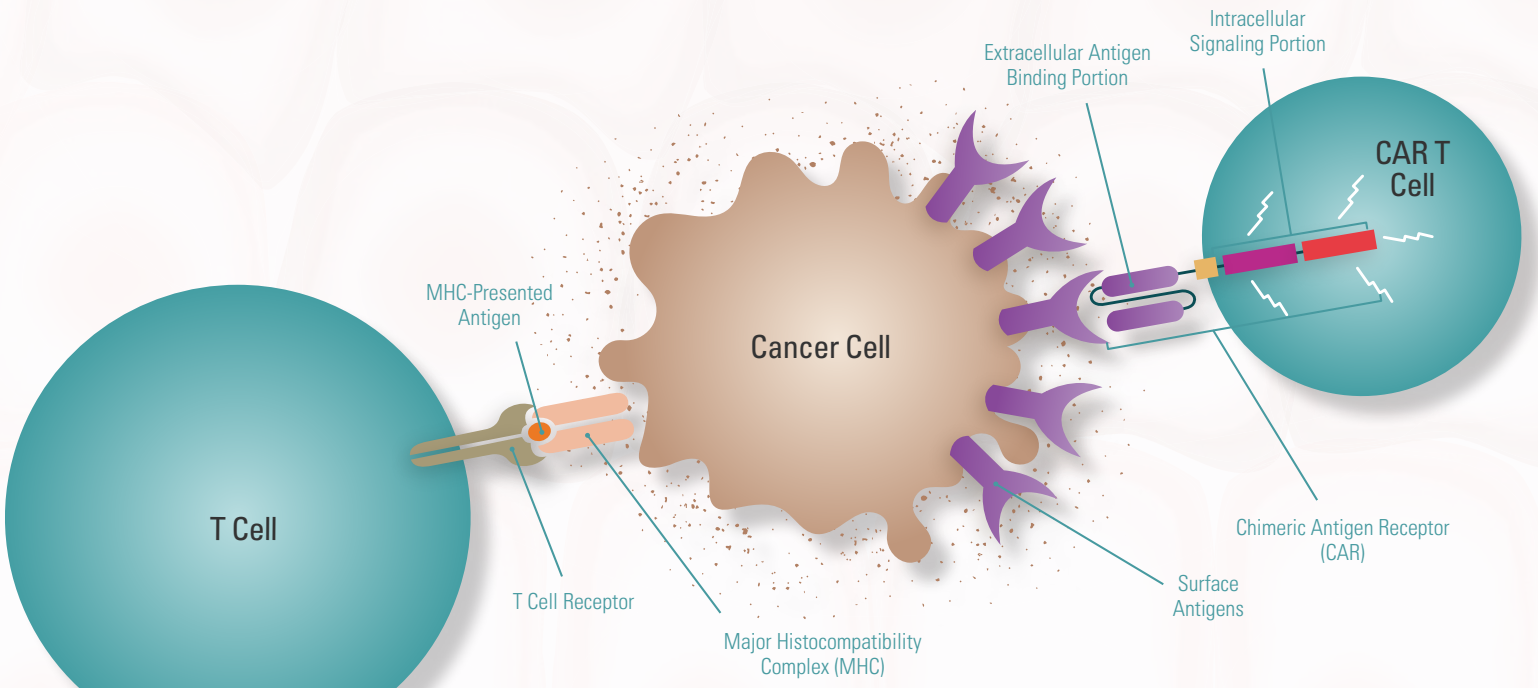
BY SARAH ANDERSON, PHD · ILLUSTRATED BY EMILY LAVINSKAS

Treating cancer traditionally relies on chemotherapeutic chemicals, radiation energy sources, or surgical procedures to eliminate cancer cells. More recently, researchers developed strategies to boost the ability of the body's immune system to detect and kill cancer cells from the inside. One such cancer immunotherapy, chimeric antigen receptor (CAR) T cell therapy, emerged as a promising addition to the arsenal against cancer. From initial drug design to treatment protocols, researchers are finetuning CAR T cell therapy to improve outcomes for cancer patients.

1 What are CAR T cells?

As white blood cells involved in the immune system's response to foreign substances, T cells patrol the body for cells housing viral pathogens or cancer-causing genetic mutations (1). T cells express receptors that bind to antigens loaded onto the major histocompatibility complex (MHC), a protein complex on the surface of the infected or cancer cell that helps the immune system detect the invader (2,3). Upon binding to the MHC-presented antigen, the T cell initiates cytotoxic pathways that trigger the aberrant cell's death (2,3). However, in order to evade the immune system, cancer cells can alter gene expression or protein function to downregulate MHC on the cell surface, preventing T cells from recognizing and effectively clearing them (3,4).

Due to specific genetic mutations or changes in gene expression, cancer cells can uniquely express certain antigens on their surface (5). Scientists modify T cells with CARs, synthetic proteins that bind to these antigens, enabling T cells to access cancer cells through a back door (2,3). The extracellular portion of the CAR binds to a cancer cell surface antigen, anchoring the T cell to its unsuspecting target (2,3,6). The intracellular portion of the CAR transmits and amplifies a signal that activates the T cell's cytotoxicity, killing the cancer cell (2,3,6).

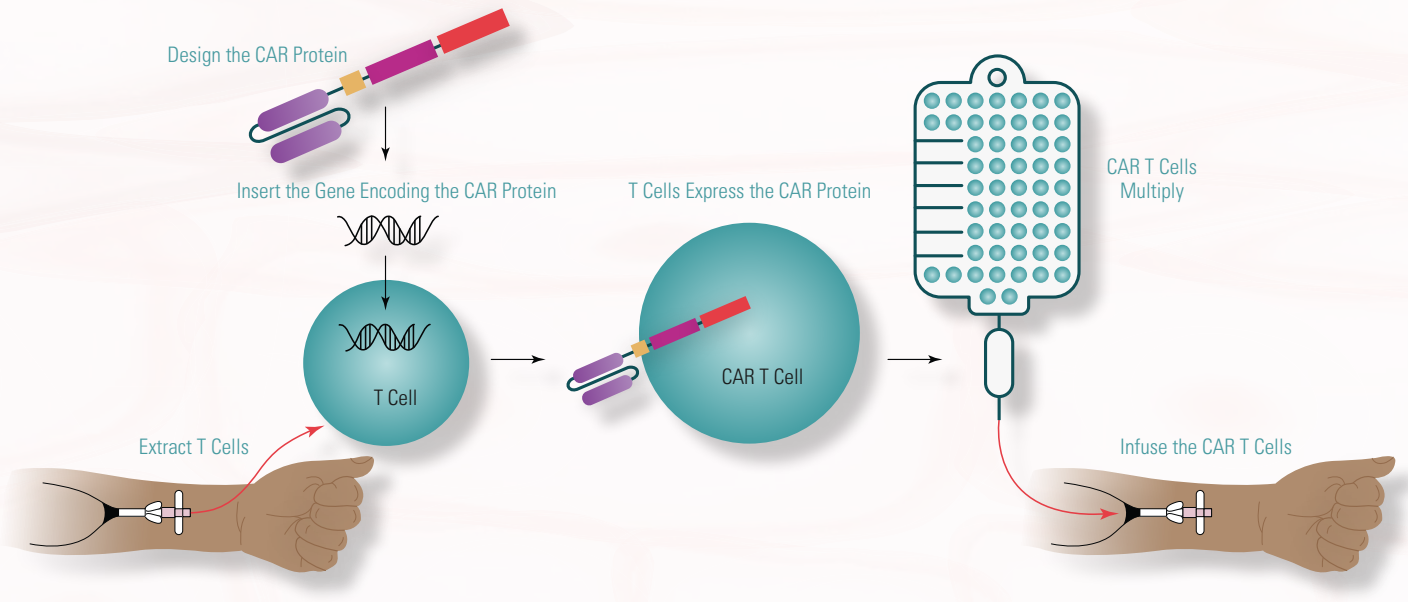


2 How do scientists engineer CAR T cells?

To make a CAR T cell, scientists must first design the CAR protein. The intracellular portion usually derives from natural signaling domains that activate T cell cytotoxicity, although the number and type of domains can vary (7). Researchers tailor the extracellular portion to bind to a specific surface antigen on a cancer cell (7). They employ a variety of methods, including protein engineering, computational modeling, and high throughput screening, to develop CARs with sufficient affinity and selectivity for the target antigen, stability, and ability to activate the T cell (7).

To incorporate the CAR into T cells, scientists identify the gene that provides the instructions for making the CAR protein. Using various genetic engineering approaches, they insert the gene into T cells extracted from a patient's blood, causing the cells to express the CAR protein (6). Scientists grow these CAR T cells in the lab to generate millions of them to infuse into the patient (6). While the exact number of CAR T cells a patient receives depends on how many are initially retrieved and how efficiently the modified cells multiply, one FDA-approved CAR T cell therapy requires 0.2 to 5 million CAR T cells per kilogram of patient weight (8).

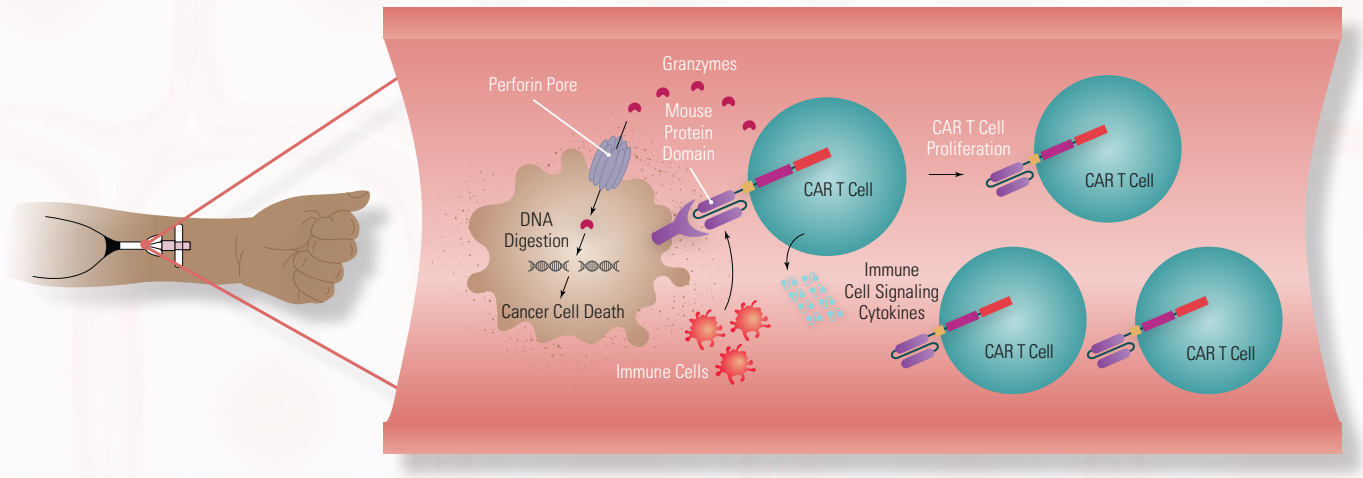
Researchers are also interested in manufacturing CAR T cells from healthy T cell donors rather than cancer patients. As the process of creating CAR T cells can take weeks, using donor cells provides a stored inventory of CAR T cells that are immediately available when cancer patients need them (6,9). Additionally, as combatting cancer can leave patient T cells exhausted, T cells from healthy donors may be able to kill cancer cells more effectively than those from cancer patients (10,11). However, donor-derived T cells may recognize the recipient's healthy cells as foreign and attack them (10). Researchers use gene editing techniques such as CRISPR/Cas9 to eliminate receptors involved in recipient cell recognition from donor T cells (10). Once CAR T cells from patients or healthy donors find themselves inside the body, their journey toward treating cancer begins.



3 What happens when CAR T cells enter the body?

CAR T cells injected into the body circulate in the bloodstream, hunting down cancer cells that present their complementary antigens. Once a CAR T cell hones in on its target, it binds to the antigen and launches its cytotoxic mechanisms at the cancer cell. The T cells release perforin proteins that poke holes in the membrane of the cancer cells and granzyme enzymes that use those holes to enter the cell (12,13). The granzymes activate a cascade of enzymes that causes DNA digestion, leading to cell death (12,13). CAR T cells can also harness T cells' natural ability to release cytokine chemical messengers to activate other immune cells to support their attack against cancer (12,13,14).

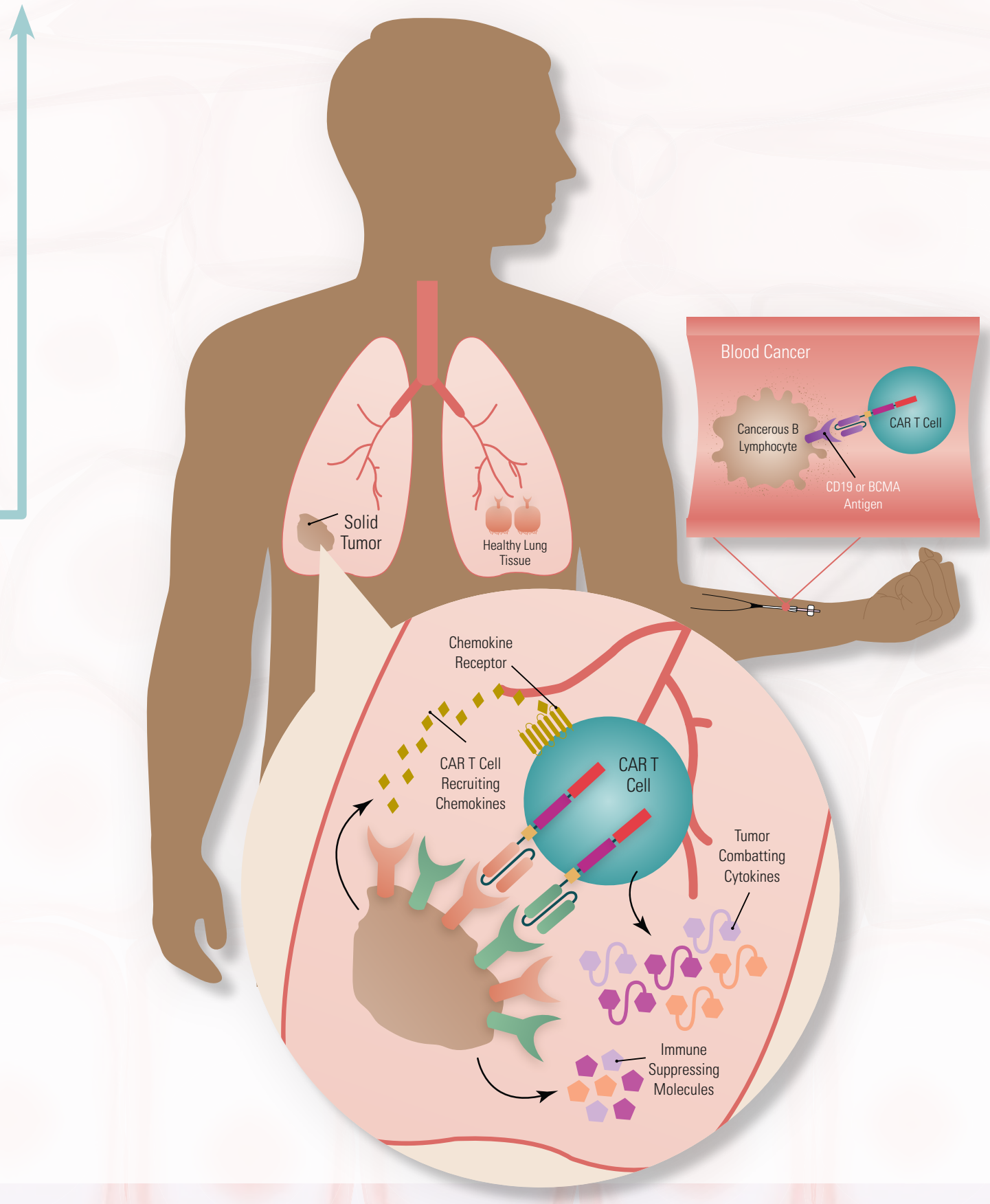
Binding to the cancer cell cues the CAR T cell to begin proliferating inside the body, creating a renewable therapy that could last years (15,16). However, the lifespan of CAR T cell therapy can be cut short if the immune system detects the CAR protein as foreign and degrades the CAR T cells (16). The source of the CAR protein plays a role in immune recognition since the extracellular portion often derives from a well characterized but unfamiliar mouse antibody (7). Researchers at the National Cancer Institute found that swapping a CAR's antigen binding domain from a mouse to a human version yielded higher levels of CAR T cells over time, suggesting that CARs made up of human protein fragments may better avoid immune-based degradation (17).



4 What types of cancer respond to CAR T cell therapy?

All six FDA-approved CAR T cell therapies treat blood cancers such as leukemia, lymphoma, and myeloma (6). These CAR T cells bind to the cluster of differentiation 19 (CD19) antigen or B cell maturation antigen (BCMA), which are robustly and selectively expressed on B lymphocyte white blood cells (6,18,19). Scientists have had less success identifying similar antigen targets on solid tumors such as those found in brain and lung cancer. Different tumors, or even individual cells within a single tumor, may express antigens nonuniformly (6,20). These antigens often exist at low levels on healthy cells, which can side track the CAR T cell's cytotoxicity (21). The unique environment of a solid tumor also secretes immune suppressing molecules that can disarm T cells and presents physical barriers that block CAR T cell penetration (6,20).

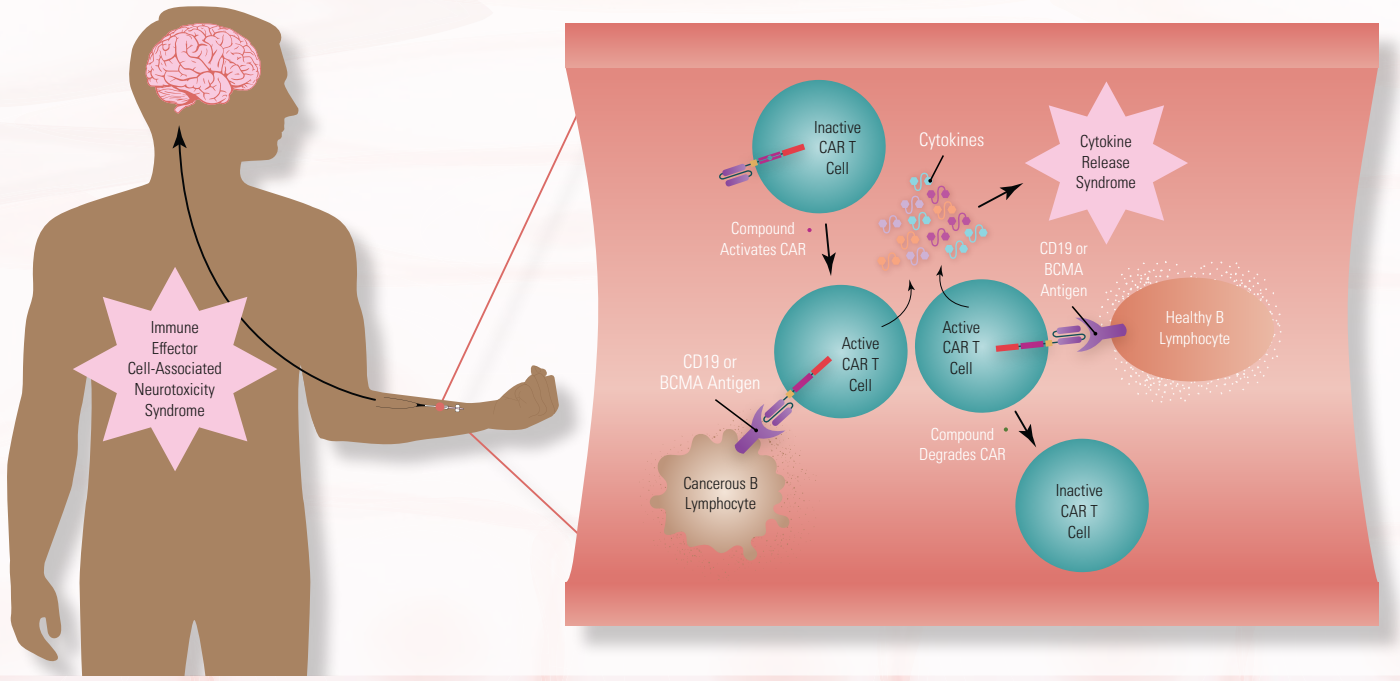
Researchers are exploring strategies to overcome these obstacles and expand the range of cancers that CAR T cell therapy can treat. They developed T cells that express two CARs that each bind a distinct antigen. The CAR T cell may initiate cytotoxicity if either antigen is present, allowing it to target heterogenous tumors, or only if both antigens are present, allowing it to better discriminate between cancerous and healthy tissues (20,22). Researchers also engineered CAR T cells to release cytokines that combat tumors' immunosuppressive mechanisms and express receptors for chemokines, immune cell recruiting molecules released from tumors, to enhance infiltration into solid tumors (16,23,24).



5 What are the side effects of CAR T cell therapy?

While T cell production of cytokines can enhance the activity of CAR T cells, it also creates the risk for cytokine release syndrome (CRS) during CAR T cell therapy. CRS occurs when CAR T cells overwhelm the bloodstream with cytokines, producing a dangerous inflammatory response that can cause a high fever, low blood pressure, and other potentially life-threatening symptoms (6,25). Cytokines may also play a role in immune effector cell-associated neurotoxicity syndrome (ICANS), another possible side effect of CAR T cell therapy characterized by neurological impairments such as confusion, seizure, and slurred speech (6,25). Both healthy and cancerous B lymphocytes express CD19 or BCMA antigens, so current CAR T cell therapies for blood cancers can kill off normal white blood cells, impairing the body's ability to fight infection (6,18,19).

While clinicians can manage some of these side effects with drugs, researchers want to reign in CAR T cell toxicity by designing cells where activity can be switched on and off by various stimuli (21). For example, researchers at the University of California, San Francisco designed a CAR in which two components assemble to form a functional protein only in the presence of a small molecule (26). Similarly, researchers at the Dana-Farber Cancer Institute and Massachusetts General Cancer Center engineered a CAR protein that degrades upon interaction with a chemical compound (27). Researchers can turn CAR T cells on or off by administering these exogenous drugs, providing a form of control over CAR T cell dosage that may reduce side effects (21).



Putting the CAR T before more chemotherapy

CAR T cell therapy is still in its infancy with its first FDA approval in 2017 (6). At present, many oncologists approach CAR T cell therapy as a last resort for treating cancers after more established forms of treatment have failed (6). Chemotherapy remains the first line of defense against blood cancer, but in two recent clinical trials, researchers found that patients with non-Hodgkin lymphoma who received CAR T cell therapy as a second line treatment showed better prognoses than those who underwent a standard regimen involving additional rounds of chemotherapy (6,28,29). In another ongoing clinical trial, researchers are assessing the efficacy of CAR T cell therapy in children and young adults with acute lymphoblastic leukemia whose cancer did not respond to initial chemotherapy (6,30). These studies will help determine whether some patients may benefit from undergoing CAR T cell therapy earlier. With efforts to optimize CAR T cell manufacturing, extend CAR T cell lifespan in the body, translate CAR T cell advances to solid tumors, and improve CAR T cell safety underway, CAR T cell therapy will continue to cement itself as a new frontier in cancer treatment.

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disease pathways

To Treat Parkinson's Disease, Start in the Gut

New research is unraveling the connection between the gut microbiome and Parkinson's disease, revealing potential gut-targeted therapeutic strategies.

BY STEPHANIE DEMARCO, PHD

Sarkis Mazmanian studies how gut microbes contribute to the pathogenesis of Parkinson's disease.

CREDIT: CALIFORNIA INSTITUTE OF TECHNOLOGY

YEARS BEFORE THE MUSCLE stiffness and tremors typical of Parkinson's disease emerge, a seemingly innocuous symptom appears in most people who will eventually be diagnosed with the disease: constipation.

Scientists have known about the connection between gut trouble and Parkinson's disease for hundreds of years. In his initial description of the disease, James Parkinson himself wrote of a patient in the late stages of the disease, "The bowels, which had been all along torpid, now, in most cases, demand stimulating medicines of very considerable power" (1).

Constipation symptoms typically begin 20 or more years before Parkinson's disease's characteristic motor symptoms (2). This early onset of gut symptoms, which occur in about 80 percent of Parkinson's disease patients, made many researchers wonder if the gut microbiome plays a role in the disease (3). An explosion of recent research points toward yes.

"What really struck me is the level of consistency between studies, between cohorts in Parkinson's," said Sarkis Mazmanian, a gut microbiome researcher at the California Institute of Technology. "The same group of organisms is depleted. The same group of organisms is increased in [Parkinson's disease] relative to controls. And I highlight this because this is not the norm in microbiome analysis. If you look across autism, you look across Crohn's disease, you look across any other disorder where the microbiome has been implicated, I personally haven't seen this level of consistency."

As researchers probe this gut-Parkinson's disease connection, new data continue to reveal the strength of this relationship. By using animal models and profiling human microbiomes, researchers uncover the molecular mechanisms and microbial species that may contribute to Parkinson's disease, leading to potential gut-targeted therapies.

Nothing stays in vagus

In Parkinson's disease, alpha-synuclein proteins form aggregates in neurons in the brain. Dopaminergic neurons are particularly vulnerable to alpha-synuclein build up, and their progressive loss is one of the hallmarks of the disease. While both genetics and environmental aspects contribute to Parkinson's disease, no one combination of factors causes the disease.

In 2003, the neuropathologist Heiko Braak, then at the University of Frankfurt, put forward an intriguing hypothesis for a Parkinson's disease cause (4). Because the gut connects to the brain via the vagus nerve, Braak wondered whether a pathogen originating in the gut could lead to the hallmark features of Parkinson's disease: aggregation of alpha-synuclein proteins in dopaminergic neurons and their subsequent degeneration.

CREDIT: DANIELLE MOR

Danielle Mor uses* C. elegans *to study the early stages of Parkinson's disease.

Since then, researchers reported that alpha-synuclein expressed in neurons that emanate from the gut — called enteric neurons — can travel via the vagus nerve to the brain in mice and rats (5,6). In humans, scientists saw that alpha-synuclein was expressed in colon tissue taken from three people two to five years before doctors diagnosed them with Parkinson's disease, while healthy controls expressed none (7). Adding to that, people who had undergone a procedure called a truncal vagotomy — cutting off the connection between the vagus nerve and the stomach — had a significantly reduced risk for developing Parkinson's disease compared to the general population (8).

> "If we understand what's happening in the GI tracts of people who are going to then develop Parkinson's disease and have this unfortunate, devastating degeneration, we can maybe start to help people early on."
>
> *– Danielle Mor, Augusta University*

Numerous studies profiling the gut microbiome noted clear differences between the microbiome composition in healthy people versus people with Parkinson's disease. But some of the strongest evidence linking the gut microbiome to Parkinson's disease arose when Mazmanian and his team studied a mouse model of Parkinson's disease that overexpresses alpha-synuclein in its neurons (9). When Mazmanian's team depleted the gut microbes from Parkinson's disease mouse models, the animals' motor functions improved, and they had decreased alpha-synuclein aggregates and neuroinflammation in their brains. Then when the researchers transplanted microbiomes from humans with Parkinson's disease or heathy controls into their mouse models, the mice with the human Parkinson's disease microbiome showed worse motor symptoms.

The results suggested that "the microbiome plays a role in the mouse model, but also much more tenuously, suggested that there may be a functional role for the human microbiome in Parkinson's," added Mazmanian. But proving this functional role is no easy task.

A seed of gut alpha-synuclein

If Parkinson's disease starts in the gut 20 or so years before motor symptoms begin, how can researchers determine who will eventually develop Parkinson's disease and study those early gut stages? The answer that Yoon-Seong Kim, a Parkinson's disease researcher at Rutgers University, landed on starts with giving mice an enema of rotenone — a pesticide outlawed for agricultural use in the United States since 2007.

Rotenone is a potent insect and fish-killer, and it's not great for humans either. In fact, researchers reported that farm workers exposed to rotenone had a higher incidence of Parkinson's disease (10).

"I hypothesized that rotenone mediated some condition… that caused the microbiome change, or directly caused some gut condition that may feed back to affect the microbiome," said Kim. "We want to investigate the molecular mechanism of how alpha synuclein… initially aggregates in the gut. What kind of cell is responsible for initial aggregate alpha synuclein in the gut?"

Kim and his team developed a rotenone-based mouse model of Parkinson's disease, which they presented at the 2022 Society for Neuroscience meeting (11). After taking an initial gut microbiome sample, the researchers flushed the mouse colon with rotenone daily for six weeks and then took another microbiome sample. They let the mice live a normal life for seven months and then took a final sample of their gut microbes.

When Kim and his colleagues compared the six week and 28 week microbiome samples, they were shocked. "The microbiome change right after six weeks is minimal," he said. "But when you leave the animal for an additional seven months, we can see the clear difference in the microbiome."

The rotenone-treated mice accumulated alpha-synuclein aggregates in the gut mucosa and in the neurons that connect the gut to the brain. The composition of bacteria in their gut microbiomes also changed. Bacterial taxa that exist at increased levels in the guts of people with Parkinson's disease — *Lactobacillus* and *Bifidobacteria* — both increased in rotenone-treated mice.

The detrimental effects did not just stay in the gut. The researchers tested the mice's motor skills by having them walk on a rotating wheel. The rotenone-treated mice fell off the wheel much sooner than the controls. When the team dissected the mouse midbrains, the rotenone-treated mice had lost more dopaminergic neurons than control mice.

"We have some proof directly testing gut microbiome changes with this model," said Kim. "That is the starting point."

Now that they have a model that recapitulates the classic Parkinson's disease characteristics, Kim and his team are ready to explore it. Their first goal is to identify the alpha-synuclein aggregate producing cells in the gut and to find a gut-targeted therapy to prevent it.

They are also following these animals for longer than seven months to see if they develop cognitive impairments associated with Parkinson's disease, and since Parkinson's disease is one of old age, they want to see how setting up the rotenone treatment model in older mice affects their microbiomes and Parkinson's disease pathology.

"We know we can prevent Parkinson's disease pathogenesis, so that is the thing that everybody's working on. There is a reason why we need some good models to test," he said.

The lactobacillus and the worm

Living on a solid diet of only bacteria, the roundworm *Caenorhabditis elegans* is an ideal model system for studying the gut microbiome and disease. A surprising challenge, though, is getting the worms to eat bacteria they've never seen before.

"They definitely have their preferences," said Nicole Johnson, a graduate student studying the role of the gut microbiome in Parkinson's disease in Danielle Mor's laboratory at Augusta University.

CREDIT: DANIELLE MOR

Nicole Johnson, a graduate student in Danielle Mor's laboratory, feeds worms different kinds of bacteria to see how they influence the development of Parkinson's disease.

Like Kim, Mor and Johnson want to use the their picky-eater worms to uncover how Parkinson's disease begins.

"If we understand what's happening in the GI tracts of people who are going to then develop Parkinson's disease and have this unfortunate, devastating degeneration, we can maybe start to help people early on," said Mor.

"In the literature from Parkinson's patients, *Lactobacillus* species are increased [in the gut], and it's just not known if that's playing any direct role in the disease. So, we really want to investigate that."

In a poster presentation at the 2022 Society for Neuroscience meeting, Johnson reported that when she fed the bacterial species *Lactobacillus brevis* — a species increased in the guts of people with Parkinson's disease — to *C. elegans* expressing human alpha-synuclein in their muscles or neurons, a whole host of things went wrong (12).

> "That was just a really fun science moment, just in general, because it did something completely opposite of the initial expectation but still gave really promising data on another lead to follow."
>
> *– Nicole Johnson, Augusta University*

Compared to the worms fed a standard diet of *E. coli*, the *L. brevis* fed worms did not move as well, and they had reduced dopaminergic neuron function. Johnson and Mor also saw that cholinergic neuron function decreased in the *L. brevis* fed worms.

"There's a huge focus on dopamine neurons because they are absolutely an important neuron type in Parkinson's disease, and the loss of those cells causes motor defects. But actually, there are other cell types that die in Parkinson's disease," explained Mor. The decreased signaling in cholinergic neurons may contribute to the decrease in motor function.

Mor and Johnson's biggest surprise came when they looked at alpha-synuclein aggregation in the *L. brevis*-fed worms.

"There's actually a decrease in the number of large aggregates," Johnson said. "That was definitely really shocking, but also really interesting because it went the opposite direction from what we had anticipated. So, then we went back, [and] we're like, well why?"

When they looked more closely, Mor and Johnson saw that the *L. brevis* fed worms had an increase in soluble alpha-synuclein. This soluble fraction could contain nontoxic alpha-synuclein monomers or small aggregates or oligomers of alpha-synuclein, which can be quite damaging to the body.

"There's a big field of oligomer research showing that those small aggregates are pretty toxic, and there's kind of a debate as to whether or not oligomers are the aggregate species that we should be targeting for therapy," said Mor. "This is part of the larger question of what protein aggregation is even doing in these diseases," she added. "Across diseases — Alzheimer's, Parkinson's, Huntington's, ALS — everybody is trying to understand the role of protein aggregates in neuron death, and it's just not clear."

Johnson and Mor plan to use liquid chromatography to determine whether there is an increase in alpha-synuclein monomers or toxic oligomers in the worms fed *L. brevis* and to better understand how different sized alpha-synuclein aggregates may contribute to Parkinson's disease.

When researchers fed mice with Parkinson's disease a high fiber diet, they moved better and showed fewer Parkinson's disease symptoms.

"If we had seen what we had expected, we probably wouldn't have gone down the route of trying to understand oligomers," said Mor. Johnson added, "That was just a really fun science moment, just in general, because it did something completely opposite of the initial expectation but still gave really promising data on another lead to follow."

Some fiber a day keeps Parkinson's disease away

Eating a high fiber diet has all kinds of beneficial effects. Fiber promotes good bowel health, helps maintain blood sugar levels, and reduces cholesterol levels, just to name a few. It turns out that a high fiber diet also associates with a decreased risk for Parkinson's disease (13).

Mazmanian wondered if increasing dietary fiber might have beneficial effects in a mouse model of Parkinson's disease.

"Fiber can be broken into many things, one of which is short chain fatty acids," he said. "One of the things they do is really modulate the immune system in the gut in ways that appear to be quite protective."

Signals from the gut travel via the enteric nervous system and communicate with microglia, which are immune cells in the brain. In neurodegenerative diseases, including Parkinson's disease, researchers hypothesize that microglial activity becomes improperly activated, leading to increased neuroinflammation which is characteristic of neurodegenerative diseases.

Mazmanian and his team fed mice overexpressing alpha-synuclein a high fiber diet for 17 weeks. When they compared the microbiomes, the alpha-synuclein overexpressing mice fed the high fiber diet had microbiomes that looked more similar to healthy ones (14). The mice also had improved motor abilities and reduced alpha-synuclein aggregates and microglial activation in the brain. In particular, they saw an increased expression of anti-inflammatory genes in microglia of mice fed a high fiber diet, suggesting that the diet shifted microglia into a more neuroprotective state.

CREDIT: YOON-SEONG KIM

With his new mouse model for the beginning stages of Parkinson's disease, Yoon-Seong Kim studies the connection between the gut microbiome and alpha synuclein aggregation.

"Even though we got a beneficial effect, an exciting result, we wanted to know, is it because of microglia?" Mazmanian said.

He and his team then depleted microglia from the mice. Even when they fed these mice a high fiber diet, the mice without microglia showed none of the beneficial effects, indicating that microglia are necessary for the good effects of the high fiber diet.

"What the study did not reveal is how. What are microglia doing differently in the context of dietary fiber that makes them more protective than microglia that are not exposed to short chain fatty acids?" Mazmanian asked. He and his team plan to investigate that mechanism in follow up studies.

For now, this study is the first that shows that a dietary intervention proffers clear benefits in a mouse model of Parkinson's disease, adding to the other potential benefits of eating a high fiber diet.

"There are just so many benefits to dietary fiber, and just in your normal everyday life, let alone staving off a disease that may be 20, 30 years away," Mazmanian said. But getting people to stick with a high fiber diet is hard.

"People like to take pills. They don't like to change their dietary habits over long periods of time," he said. "But again, it introduces another way that one can intervene through the microbiome, and it's not by fecal transplants or by probiotics, but by diet — using diet as the actual medicine." ■

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Tackling Metabolic Disease

A new oral bead technology triggers glucose release in discrete parts of the gut to restore metabolic balance.

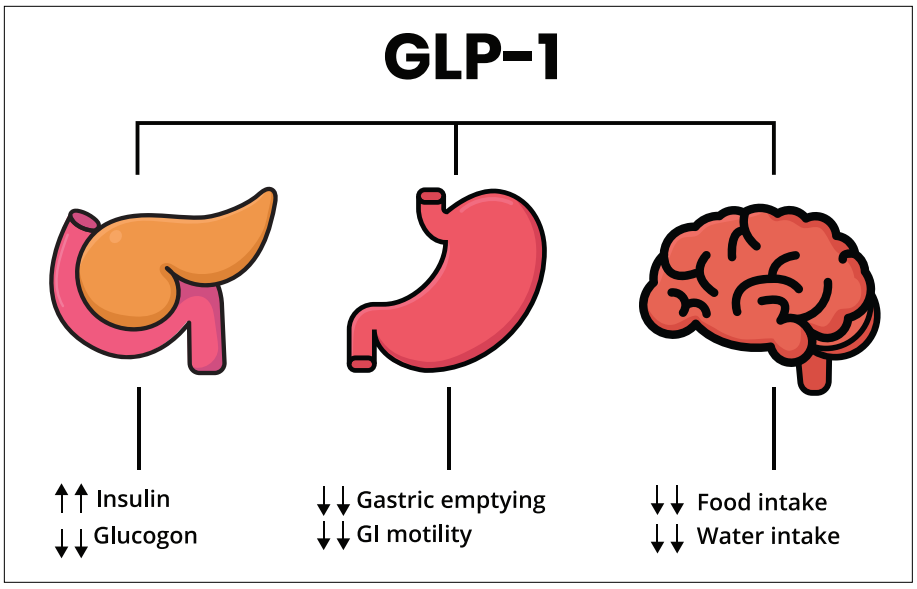
BY DANIELLE GERHARD, PHD

OBESITY AFFECTS MORE than 800 million people worldwide and drives conditions such as type 2 diabetes and cardiovascular disease (1). In recent years, the treatment landscape for metabolic disease, particularly type 2 diabetes, has burgeoned. Synthetic drugs designed to target key signaling pathways in the gut effectively reduce blood glucose levels and restore insulin signaling. However, researchers at the global biopharmaceutical company Aphaia Pharma take a different, more natural approach to improve metabolic health. Their approach may well pay off as preliminary findings from a Phase I clinical trial suggest that their novel drug may be a viable alternative to more invasive procedures such as gastric bypass surgery.

Moving down the tract

While relaxing after a tasty meal, the human gut is hard at work. As food and liquids travel from the stomach through the upper and lower parts of the small intestine, they leave valuable nutrients in their wake. Gut function varies along this path, with upper regions driving nutrient absorption and passing along leftover food particles to lower regions that orchestrate nutrient sensing.

The gut reduces that slice of pizza to the basic building blocks of nutrition. Carbohydrates become simple sugars; proteins break down to amino acids; and fats transform into fatty acids and glycerol. These nutrients trigger the release of gut hormones that rapidly communicate information with other organs, including the pancreas, liver, and brain to regulate insulin release, blood glucose levels, and feelings of satiety.



GLP-1, a prominent gut hormone, is the target of many synthetic drugs on the market for the treatment of type 2 diabetes and obesity given its role in decreasing blood sugar levels and enhancing insulin secretion. The natural formulation APH-012 rapidly increases GLP-1 serum levels.

Dampened nutrient signaling contributes to the disrupted metabolic functioning seen in people with obesity and metabolic diseases such as type 2 diabetes (2). While deficits in blood glucose regulation and satiety are present in these patient populations, the exact causes remain unknown. Some studies suggest that a majority of food nutrients get absorbed in the upper small intestine, depriving the lower small intestine of the nutrients it needs to trigger release of gut hormones. Alternatively, a variety of factors including a high fat diet, genetics, and environmental exposures may fundamentally change how cells in the gut respond to available nutrients. Either way, a common denominator across theories of metabolic imbalance is that hormone signaling is disrupted.

Following their release, gut hormones receive marching orders to infiltrate different downstream signaling pathways. Two hormones in particular, glucagon-like peptide-1 (GLP-1) and glucose-independent insulinotropic polypeptide (GIP), help lower blood glucose by stimulating insulin release and inhibiting glucagon release (3). Over the last decade, drugs targeting these gut hormones, specifically GLP-1 receptor agonists, have gained popularity for treating type 2 diabetes. Some GLP-1 receptor agonists also lead to more than 20% weight loss, making them an attractive candidate for treating obesity and comorbid type 2 diabetes (4).

In addition to behavioral and lifestyle interventions, gastric bypass surgery is an effective treatment for some individuals with obesity.

Shrinking and rewiring the stomach to connect it directly to lower regions of the small intestine not only helps reduce food intake, but also dumps nutrients on top of the nutrient sensing cells that may otherwise be deprived of these signals. In line with this theory, gastric bypass almost immediately increases levels of key hormones like GLP-1 and GIP (3).

Despite the promise of these treatments, side effects including nausea, gastrointestinal discomfort, and elevated heart rate often lead to treatment discontinuation, raising concerns about their long-term use (5).

A simple approach to a complex system

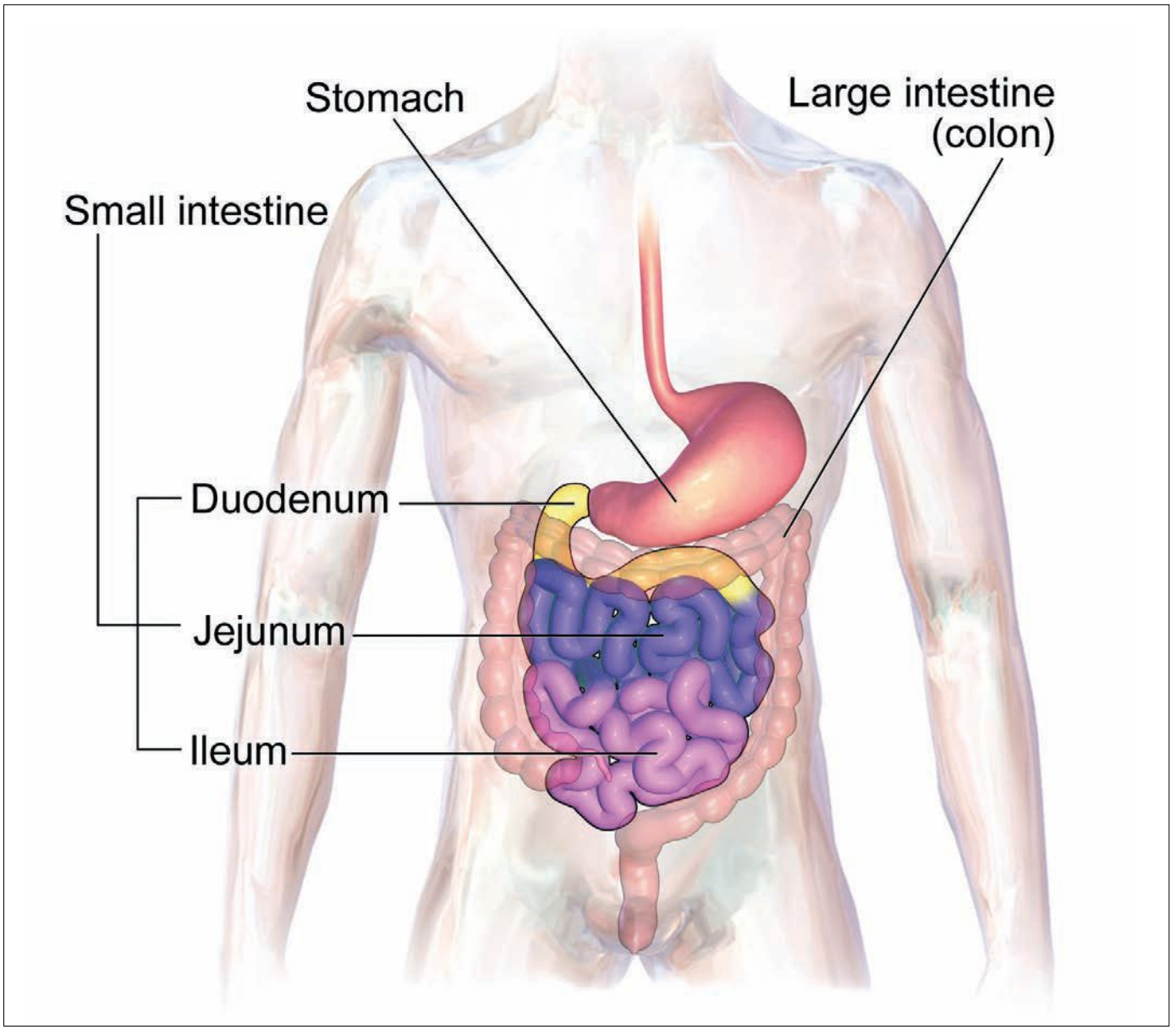
In experiments conducted in the 1960s, scientists observed that oral administration of glucose better increased plasma insulin levels compared to intravenous administration, highlighting the strong connection between the gut and the insulin-producing pancreas (6). Glucose delivered directly to lower parts of the small intestine also better stimulates insulin release and secretion of the gut hormones GLP-1 and GIP compared to glucose delivered higher up the tract (7).

This literature inspired scientists at the global biopharmaceutical company Aphaia Pharma in their quest to find a natural approach for the treatment of metabolic disease. The research team developed APH-012, an oral bead formulation designed to transport glucose to precise regions of the small intestine. Although the nuts and bolts of the technology remain proprietary, Aphaia Pharma scientists designed tiny coated beads that release natural substances specifically to the lower parts of the small intestine. "We can bypass everything that's happening in the upper small intestine that prevents nutrient sensing cells from exposure to food with a formulation that drops food on top of those cells," said Steffen-Sebastian Bolz, the chief scientific officer of Aphaia Pharma.

In contrast to most GLP-1 receptor agonists, which target just one hormone, the team at Aphaia Pharma hoped that exposing the nutrient sensing cells of the lower small intestine to glucose would result in a broader shift in metabolic response. "It's a good approach," said Frank Duca, a gastrointestinal biologist who is not involved with the research but studies gut hormones and gut-brain signaling at the University of Arizona Cancer Center. "It's a more natural, endogenous approach to increase GLP-1 levels versus GLP-1 receptor agonists, which is going to be more physiological."

Aphaia Pharma recently completed a Phase I trial of APH-012 in 20 individuals with obesity. Following a single dose of the drug, the researchers observed a broad-spectrum increase in serum levels of important gut hormones, including GLP-1. In 90 percent of patients, they observed these effects within one hour of administration, evidence of low variability in this technology's pharmacokinetic profile. Additionally, patients receiving APH-012 did not report any adverse side effects. The research team is currently preparing the complete findings from the Phase I trial for publication, which they hope will occur sometime this year.

Bolz described the nutrient sensing cells of the lower intestine as Sleeping Beauty awaiting her prince, APH-012, to reactivate dormant physiological mechanisms and restore metabolic balance. The Phase I findings are promising and suggest that APH-012 pharmacologically mimics aspects of gastric bypass while avoiding the invasive surgical procedure. Furthermore, designing a drug that combines multiple agonists to target the sundry gut hormones activated following a meal poses a challenge that oral glucose appears to meet.



The small intestine is comprised of 3 distinct regions: duodenum, jejunum, and ileum. The upper small intestine (primarily the duodenum and jejunum) specialize in nutrient absorption, whereas the lower small intestine (primarily the ileum) uses the remaining nutrients to release gut hormones such as GLP-1 that are important for mediating blood glucose and insulin signaling.

The proof is in the Phase II trials

Aphaia Pharma is in the process of recruiting 150 adult patients with obesity for a Phase II proof-of-concept study. The single dose used in the Phase I trial was too brief to measure any meaningful clinical endpoints. In contrast, the Phase II trial will follow patients taking a daily dose of APH-012 for either six months or a year. The researchers hope to see significant changes across a wide variety of measures of metabolic health, including weight, insulin resistance, fasting plasma glucose, cholesterol, and fatty liver disease.

Aphaia Pharma has big plans for their small drug. A second Phase II trial is currently in the pipeline to determine APH-012's impact on glucose tolerance in patients categorized as prediabetic. Nearly a third of American adults have blood sugar levels hovering just below the threshold required to be considered type 2 diabetes. The good news is that prediabetes can be reversed and Aphaia Pharma is optimistic that APH-012 can step in and give a gut punch to blood glucose and delay or prevent the emergence of type 2 diabetes following detection of early warning signs.

These studies are critical for determining APH-012's potential in the clinic. GLP-1 receptor agonists are quickly hitting the market as an effective means of controlling insulin and reducing weight. In part, these drugs are so effective because they deliver synthetic GLP-1, which has a significantly longer half-life than endogenous GLP-1. "A lot of the effects attributed to GLP-1 receptor agonists for weight loss are likely due to central mechanisms in the brain," said Duca. "It's unlikely that increasing endogenous GLP-1 is going to be able to reach the brain to have the same effects on weight loss that the agonists will."

Researchers at Aphaia Pharma hope that the enhanced safety profile of their natural approach will give them an edge in an increasingly competitive market. With a lower risk of adverse events, APH-012 should have higher treatment compliance and reach a wider patient population. For this reason, the researchers think that the oral bead formula could be particularly effective for treating younger populations. Childhood obesity is pervasive, and scientists predict that it will increase by 60 percent over the next decade, reaching 250 million by 2030 (1).

"This is why we think that APH-012 can be disruptive, and it can really change the treatment landscape," said Bolz. ■

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