Mark Gladwin is a scientist with something to leverage: a fundamental biological discovery. And that is why he is here in Pittsburgh.

“We think we may have stumbled upon the active ingredient in the Mediterranean diet,” he reports in his office on the sixth floor of UPMC Montefiore.

Gladwin says this calmly, without much fanfare, despite the magnitude of such a discovery. He is a youthful guy, with a head of hair just long enough that it might send another scientist to the barber. He wears snazzy shirts. He’s outgoing, and some of his colleagues wonder where he gets his energy.

“There are mysterious cardioprotective qualities to the Mediterranean diet,” he continues. “And everybody has looked...
Mark Gladwin believes maligned molecules are the cardioprotective secret to the Mediterranean diet.
at the vitamin E and the vitamin D and the vitamin C, and none of those things has panned out. But what everybody has ignored is the one molecule that is the richest but that is the bad one.”

Gladwin, an MD who came to Pitt in 2008 as a professor of medicine and chief of the Division of Pulmonary, Allergy, and Critical Care Medicine, stumbled upon the power of this “bad molecule” when he was at the National Institutes of Health. He describes the NIH as the sort of place that enabled this discovery. It provided him with a steady level of support without the need to reapply for grants every cycle. By the same token, it offered little hope for leveraging more funding for his lab after a major discovery, such as a key element of the Mediterranean diet—nitrate.

Nitrate has long been seen as belonging to the dark side of biochemistry.

It is considered a pollutant in this country, and the federal government tightly regulates the amount of nitrate in drinking water. Your bottled water is most likely nitrate-free. Municipal water suppliers have become adept at limiting our exposure to nitrate. Over a recent five-year period in Pennsylvania, for example, only a relative handful of communities in the state—with a total population of less than 70,000—had nitrate levels in their drinking water that exceeded the national health-based limits.

The negative press that nitrates receive actually pales in comparison to that of its sibling molecule, nitrite. Both molecules have been indicted because they can lead to the formation of carcinogens called nitrosamines; yet it’s unclear if nitrosamines from food or beverages actually lead to cancer in humans.

Our bodies convert dietary nitrate into nitrite. And it’s the nitrite that is biologically active and, Gladwin believes, cardioprotective.

Yet, parents are routinely told to avoid giving children hot dogs because of the nitrates they contain.

They are encouraged to lobby school boards to remove nitrite from school lunches. There are petitions one can sign to encourage the FDA to ban nitrite.

Gladwin insists that he isn’t advocating therapeutic or even routine hot dog consumption. “But the truth is that if you were about to have a heart attack, and you ate a hot dog,” he says with raised eyebrows, “based on all our data, you should have cardioprotection.”

Welcome to the Dark Side.

What follows are the instructions for whipping up a potential wonder drug. You don’t need much—just a few ingredients that every lab already has lying around or can cheaply and easily get.

Start with an ounce of oil. Nothing exotic, just regular oleic acid. (The olive oil in your kitchen is probably 55–80 percent oleic acid, by the way.) Next, mix in some sodium nitrite, which is common enough around the lab and is really just a type of salt used to cure meat. Stir. That’s it. You’re done.

That’s the Reader’s Digest version. The recipe is actually more complicated and detailed, but it’s nothing a careful and competent chemist can’t manage. The instructions (“lipid phenylethlamination/nitration protocol”) run five pages long. A chemist adds reagents at various stages to encourage particular molecular interactions and to discourage others. He stirs the clear yellowish liquid for several hours with a spinning magnet dropped into the flask. Then he follows up with standard purification measures, such as filtering the mixture through a plug of silica gel. End result: a 99 percent pure solution of a fatty acid with a nitro group (NO2) added in the middle.

Bruce Freeman, a lanky runner, cyclist, and the UPMC Irwin Fridovich Professor and chair of the Department of Pharmacology and Chemical Biology in the School of Medicine, calls it “one-pot synthesis.” His description makes it sound as though it were mixed in the break room while he munched on a sandwich and scanned the latest issue of Runner’s World, though he later notes that his lab’s method for adding a nitro to a lipid in this way is a “tour de force in organic chemistry” that postdoctoral associate Steven Woodcock managed to pull off.

And what does this drug—this nitro-fatty acid—do?

Experiments in Freeman’s lab in mice and tissue culture suggest that it might significantly protect your heart from damage before, during, and even after you’ve had a heart attack. It looks promising for diabetes, too.

Freeman’s nitro-fatty acid occurs naturally in low abundance in living tissue, and it appears unlikely to have dramatic, toxic side effects. Quite the opposite: It appears to be safe, stable, and simple to make.

It sounds so simple that one might ask why we shouldn’t simply take some nitrite-cured meat—a nice Italian soppressata, for example—and drizzle it with olive oil. Better yet, toss with some cooked beets and serve on a bed of spinach—both rich sources of nitrate, which the body quickly converts to nitrite. Sounds tasty, doesn’t it?

Freeman himself may have unintentionally provided us with a name for this dish: One-Pot Synthesis. You are the pot. Forget the flask and the hours of stirring—why not simply slosh it around in your gut?

In 2007, Freeman and Gladwin were featured speakers at a conference at the Karolinska Institute’s Nobel Forum in Stockholm. In the place where the Nobel Committee announces the annual Nobel Prize in Physiology or Medicine, these two scientists were part of a program called “Frontiers in Medicine: The Emerging Role of Nitrate and Nitrite in Biology.”

How is it that such common, mundane, and purportedly deleterious stuff could turn out to be so biologically important? To understand the research of Freeman and Gladwin, you have to go back to the story of the 1998 Nobel Prize in Physiology or Medicine, which was awarded to three scientists who discovered the role of nitric oxide in basic physiology.

Nitric oxide was mainly known as a pollutant, because it is present in automobile exhaust and cigarette smoke. It’s a free radical and was therefore thought to be a dangerous thing to have running loose in the body, where it would initiate uncontrolled reactions with cells and important biological compounds. Nitric oxide—NO, for short—was troublesome, so the experts said.

The 1998 Nobel recognized the dramatic new understanding that NO protects the heart, stimulates the brain, kills bacteria, and dilates vessels to draw blood to wherever the body needs it. Most signaling molecules work through specific receptors in the cell membrane. NO, however, proved to be unusually powerful. Because of its small size and the fact that it is soluble in fat, it easily traverses cell membranes to regulate cell activity.

Nitric oxide is so unstable in the body that it is natural to ask what compounds might act as stable sources. That’s still an open question, and one that will ultimately have many correct answers. (Nitroglycerin, for example, has been prescribed to alleviate heart conditions for more than 100 years. Now we know the drug works by releasing NO.)

At Pitt, Freeman and Gladwin are on to two big pieces of the NO puzzle, both of which are likely to lead directly to therapy for patients.

In 1990, eight years before the Nobel Committee recognized the importance of NO, Freeman was at the University
of Alabama, Birmingham, where he and others hammered out a landmark paper for the *Proceedings of the National Academy of Sciences (PNAS)* describing a confounding observation about nitric oxide. In the lab, NO combined with highly reactive oxygen radicals to generate very toxic byproducts. Clearly, if this were happening in the body, it would be part of an inflammatory event. But that’s not what Freeman found. He says:

“When I tried to replicate those test-tube chemistry-based observations in cell or animal models, we observed that nitric oxide, rather than being pro-inflammatory, had anti-inflammatory properties.”

At this time, Freeman was about to embark, courtesy of a Fulbright Scholarship, on a monthslong stint in the lab of one of his former trainees in Uruguay. There, while trying to figure out these anomalous anti-inflammatory effects of NO in living tissue, they stumbled upon an unusual fatty acid byproduct. When they looked at a fatty acid that included a long chain of 18 carbons, for example, they discovered a nitrogen compound branching off from one of the carbon bonds—something never seen before.

For nearly 20 years now, these nitro-fatty acids (NO FAs) have been a focus in Freeman’s lab. On any given day, as many as a dozen people—postdocs, students, and early-career scientists—attack this problem in his lab from multiple angles.

Two postdocs, for example, administer NO FA to mice that have elevated blood glucose levels—the same problem that plagues humans with diabetes. This follows an important paper that Freeman and colleagues published in *Nature Structural & Molecular Biology* in 2008, showing that NO FA was perhaps a safe and natural alternative to the diabetes drug Avandia. The drug lowers blood glucose levels by activating a receptor on the surface of the cell—the receptor is like a socket that is configured to fit only a molecule with a specific shape. When a molecule binds to the receptor, it initiates a series of anti-inflammatory events. No one knew of a naturally occurring molecule that fit this receptor until Freeman’s group showed that NO FA does. Avandia’s annual global sales total in the billions, but it has laterly been associated with elevated risk of bone fracture in women, as well as with heart failure, heart attack, and death.

Freeman says the seminal paper in this area is yet to come. “The real relevance is when we are able to publish the observation that fatty acids have a beneficial, clinically relevant effect in treating sick mice,” he says. “That paper is cooking right now, in the peer-review process.”

In a small room off Freeman’s main lab, the air hums with the activity of two powerful mass spectrometers, machines capable of detecting and identifying molecules in minuscule amounts. Here, researchers learn what other molecules are created along the NO FA pathway, what tissues contain them, and how much is found there.

At a table in the main lab, Volker Rudolph, an MD and a visiting research associate in pharmacology and chemical biology, gives a heart-disease/NO FA briefing. He says that when someone experiences a blockage in the coronary artery—a myocardial infarction—blood supply to part of the heart muscle is cut off. A cardiologist can then remove the blockage and open the artery with a stent, drugs, or a balloon. As the affected area of the heart muscle is reperfused with blood, the cells there suffer further damage. More than half of the cells that will die as a result of the heart attack will die upon reperfusion. The patient will then live with a damaged heart; the rest of the muscle will have to compensate for this dead muscle tissue—the infarct. The heart may grow larger to compensate, too, and this comes with its own negative health consequences.

In a mouse model of such heart attacks in Freeman’s lab, Rudolph injects NO FA into mice just before the coronary artery is reopened—sometimes just three minutes before, mimicking the sort of intervention that a cardiologist could someday perform on a human. In his experiments, the mice treated with NO FA always have smaller infarcts than the control mice.

In other words, they have cardioprotection. It’s as if they had just sidled up to the Dark Side Café and ordered a spinach salad and a round of hot dogs.

Mark Gladwin will never forget the time that he, as a young NIH scientist, presented some of his early work on nitrite at a conference. “Ridiculed” is too strong a word, he says, but it’s worth noting that he can’t come up with a better one.

In the late 1990s, Gladwin was investigating a mysterious property of nitric oxide gas: You can breathe it, and it will lower blood pressure in the lung. Yet, according to the prevailing wisdom, the gas would not reach the rest of the body because it was unstable in the blood and would be instantaneously destroyed by reactions with hemoglobin.

“We started looking at the possibility that there were some subtle peripheral effects. We called it the ‘endocrine effect of inhaled nitric oxide’ because it was carried in the blood, distally.” They were looking for a blood-borne NO pathway.

Gladwin and colleagues used an inhibitor to block NO production in the arms of healthy volunteers. Then they gave inhaled NO gas and showed that NO was still reaching the arm. Next question: What was the source of NO in the blood?

“So then we started rounding up the suspects,” Gladwin says. “What could this species be? And I was looking at all these strange things like nitrosated albumin.”

For bookkeeping purposes, Gladwin would always measure nitrite in the blood because it was an oxidation product of NO. Everyone knew that nitrite was an inert waste product that appeared in the blood when you breathed NO gas. So measuring nitrite was a way of demonstrating that your healthy volunteer was, in fact, breathing NO gas exactly as you intended.

“I even had a technician doing the nitrite measurements because they weren’t important,” Gladwin says. “I spent day and night working on these complicated molecules that were really hard to measure, and my technician was breathing through all the nitrites.”

But a telling sign showed up when he looked at the technician’s measurements: gradients. There was more nitrite in the artery delivering blood to the arm than there was in the vein leaving the same arm. The nitrite was being consumed.

“So we published a paper in 2000 saying, ‘Look, we have this endocrine effect in the arm, and the only species we’re seeing is nitrite, and there are gradients suggesting it’s being used.’”

Is it possible, Gladwin asked, that this low concentration of nitrite is a source of NO for dilation of these blood vessels?

This is the question that Gladwin posed to a gathering of other scientists. And he recalls that a very famous scientist, of whom Gladwin was a bit in awe, stood and said, “Mark, very nice work. You’re doing really nice work. I just have one question: I use nitrite as a control in my aortic ring experiments. So how could this be active?”

And the room went silent. Gladwin strug-
Mark Gladwin (left) and Bruce Freeman with Alfred Nobel at the Nobel Forum in Stockholm.

Gladwin's team buffered the nitrite solution and measured the NO in the bag—a negligible amount. They repeated the experiment in nine new people, and they got the same result.

“Whoa!” says Gladwin. “So then we dropped the concentration by two logs—from 200 micromolar in the arm to 2 micromolar. We repeat that, and it dilates 10 of 10 people.”

A lot has happened since then, including the 2007 Nobel Forum conference on the emerging role of nitrate and nitrite in biology, where both Gladwin and Freeman spoke. Also, the famous scientist who used nitrite as a control, Gladwin reports, now says that he always knew nitrite was a vasodilator.

Gladwin published in 2005 that low doses of nitrite prevent heart attack.

“It was repeated by people all over the world,” he says. Gladwin and Pitt colleagues have proposed a heart attack trial to the National Heart, Lung, and Blood Institute.

What about the idea of nitrates and nitrites being toxins or carcinogens?

One scientist who viewed Gladwin’s early papers harshly had been convinced for years that nitrite was harmful. He had a hard time believing Gladwin’s results, and data from his own lab showed that nitrite was toxic. But he administered doses hundreds of times higher than Gladwin’s. Both Freeman and Gladwin agree that there is at least the potential, with large doses of these compounds, for genotoxicity and DNA damage.

Like all good things, nitrite is only good in the proper amount, says Gladwin, and the same probably holds true for NO, FA. He cautions that these are reactive nitrogen species that cause powerful reactions. Gladwin thinks the sweet spot for nitrite and nitrited lipids is in very low concentrations.

At Pitt, Gladwin is the first director of the Hemostasis and Vascular Biology Research Institute. In his career, he has made major contributions to understanding the role of lung complications such as pulmonary hypertension in sickle cell patients, and he has advanced the study of nitrite-based therapies for alleviating the vascular complications of sickle cell disease. In 2008, he coauthored a review of the mechanisms of sickle cell disease for *The New England Journal of Medicine* and a review of the nitrate–nitrite–NO pathway for *Nature Reviews: Drug Discovery*.

“What we’re doing is thinking of any disease where oxygen is low and nitric oxide might be useful—heart attacks, high blood pressure of the newborn,” Gladwin says. “And in all these preclinical models, the nitrite is working. So coming to Pittsburgh is very exciting for me because now I have the opportunity at UPMC to move this discovery into clinical practice. We’re limited at the NIH in patient population. Also, one of the ideal targets for nitrite is going to be solid organ transplantation, and Pittsburgh is obviously the center of the universe for that.

“In solid organ [transplants], you essentially take a healthy organ, and you remove it, and you block its blood flow for hours. We think the nitrite—well, we’ve shown that nitrite—essentially can stabilize organs in the setting of blocking blood flow. We joke that we’re turning an organ into a hot dog, but it’s a state of suspended animation where that tissue is protected from damage.”

Working with the Pittsburgh Life Sciences Greenhouse, Freeman has started up a biotech company to help bring NO, FA out of the lab, where it has only been tested in animals, and into clinical trials in humans. The potential market is significant.

“There are [University of Pittsburgh] patients covering composition of matter, method claims, and using [NO, FA] to treat specific disease conditions,” confirms Hank Safferstein, CEO of Complexa, the firm he launched with Freeman.

“We’re going to focus probably our initial work on metabolic disease, in particular type 2 diabetes. Obviously, this is a big area in terms of unmet medical needs.”

Is it also possible that a newfound awareness of these nitric oxide pathways might simply encourage dietary changes that would lead to healthier hearts?

“Look at the Mediterranean diet. It’s loaded with prosciuttos and salamis. It’s hot, so you have to cure your meat,” notes Gladwin, pointing out that such a diet also includes vegetables like beets—rich in nitrates, which the body converts to nitrite. “Also, in the Mediterranean diet, you live on well water—nitrate everywhere. In the U.S., we tightly regulate nitrate in our water. If a well has nitrate, you can’t use it. Then the American diet: uncured meat and potatoes, pastas, bread, carbs. Zero nitrate. We have a nitrate-deficient diet. Is it possible that part of the cardiovascular disaster of the Western diet is that we’ve depleted this essential mineral from our diet?”

To Gladwin, that sounds like a rhetorical question.