Pediatric Nutrition
CONTINUING EDUCATION FOR CLINICIANS

TRANSCRIPT

Biomarkers and the Prediction of Risk for NEC

Editor's Note: This is a transcript of a live presentation delivered in November 2024. It has been edited for clarity.

Misty Good, MD, MS
Division Chief, Neonatal-Perinatal Medicine
Co-Program Director, Pediatric Physician Scientist Training Program
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina

I know I don't have to tell this room what NEC is, but I do want to start the talk by saying why I've dedicated my career to studying this disease. We've all seen these babies at the bedside that are premature and doing fine and growing, with all of the amazing work that Mandy and others have done, but then suddenly develop distended abdomen, bloody stools and the next thing you know they are having surgery and are dying from NEC at the bedside. When I was a pediatric resident, I became really interested in why is this happening to these babies and what can we do to prevent it? When I became a fellow and I was in a lab and working with mice, I really wanted to know more what is happening to our babies and how can we translate what we do in the lab to the bedside. We started collecting some samples which I'll talk more about, but here are some histology slides from small intestinal samples that we've obtained from the operating room of these infants.

We all know there's so many challenges in diagnosing NEC and how we can really distinguish this from other diseases, such as feeding intolerance, etc, but I think it goes without saying that we know that the pathogenesis of NEC is really highly complex and multifactorial and several of those factors actually remain unknown. One of the ways in which we can dig into that a bit is to analyze a lot of the samples from these babies and so that will be the premise of the talk.

We know that not all NEC is the same and so, in premature infants with NEC, the very smallest babies vs the larger growing babies that are just about ready to go home, compared to the term babies that have congenital heart disease or have been exposed to substances in utero, we know that the etiology varies between patients. That makes a diagnostic dilemma for all of us at the bedside and sometimes we're diagnosing this, as you all know, when it's too late. The other thing that really had limited the field for several years is that NEC impacts a relatively small number of patients at individual centers. Now, we all know we've seen NEC and not all NEC is the same, but there's so much heterogeneity in the disease that we really need to dive into that at this particular gestational age, what does NEC look like for this or that group of patients.

What's most important is that collaboration between institutions with highly skilled and committed teams of investigators because, again, as we all know this is a 24/7 disease, and it is really required to obtain adequate samples for quality research.

The gold standard is obviously the abdominal x-ray and so, we know that there's a lot of diagnostic challenges in that regard and early detection can certainly reduce mortality and morbidity, but really remains challenging. Early studies have focused on C-reactive protein, for example, you know, is the white blood cell count going up, but also we know that babies have died in front of us with a normal C-reactive protein and we know that they have NEC. We know also that, when we look at the abdominal x-rays, we can't always see the pneumatosis and I don't know about your radiologists, but ours will say stool vs pneumatosis. We really need something to help us distinguish that. We actually call that poopatosis in my research group and we really need something else.

We have shifted towards obtaining abdominal ultrasounds in the setting of a gasless abdomen, for example, and they can be more sensitive than our x-rays and useful in clinical settings. Not all x-rays are as clear as this where there's pneumoperitoneum and extensive amount of pneumotosis, but we do need better diagnostic tests.

When we think about diagnostic tests, specifically we think about biomarkers and can there be a test at the bedside that can tell us, yes, this baby has NEC or no, they don't. There's a lot of work that's been done in this field over the last several decades, so I'm just going to give a high-level overview here about inflammatory markers.

We all know that NEC is an inflammatory disease. Babies will have elevated cytokines and chemokines and all kinds of other inflammatory markers, but it doesn't really tell you does a baby have NEC specifically or do they have another infection, for example, like sepsis, or just feeding intolerance or they're stressed about something else, for example. Then, in the last 8 to 10 years, microbial and metabolite markers have become very important. Specifically thinking about the gut microbiome, for example, which I'll talk a little bit more later, but also microbial metabolites and how can we then diagnose NEC sooner and what can we do about it more specifically. Then some more recent work is looking at a multiomic approach and our group and others have looked at this, so I'll talk a little bit more about this in the next slides, but specifically looking at proteomic markers and other metabolomic markers where specific peptides or amino acids are elevated or decreased in babies with NEC compared to their control premature counterparts. Then we have a grant looking at epigenetic markers and so I know we've talked a lot about genetics and we just don't have enough data yet to say that NEC can be

predisposed from a genetic disease. I will say there's a lot of good work going on in this area, but we don't have the final verdict on that yet. But I'll talk a little bit about some epigenetic modifiers that may be important in the future.

Drilling down on this, so inflammatory and microbial biomarkers for NEC, as I mentioned cytokines and chemokines, but there's been a lot of good work looking at what has happened in the premature infant's trajectory, specifically prior to diagnosis of NEC, and some of the cytokines specifically that have been associated with NEC severity include TNF-alpha, interleukin-6, interleukin-8 and we see this in almost all of the babies that have NEC. But what happens and what is the time course and trajectory of those cytokines really remains to be determined because we aren't analyzing a lot of these samples prior to diagnosis of NEC, only getting blood work, for example, when we think they have a diagnosis, which is often too late.

There's been a lot of good work in the microbiome again over the past 8 to 10 years and there are these shifts in microbial composition in the stool, including decreased Bifidobacterium and increased Enterobacteriaceae that correlate with NEC. When thinking about that, we can see these shifts in the microbiome 48 hours prior to a diagnosis of NEC. If we have the ability to be able to see that at the bedside in real time, what would that mean for us as clinicians and what will we be able to do about it? Could we start antibiotics earlier? Do we need to modify the microbiome with either probiotics or even probiotics so we can feed the microbiome and move those shifts around to a more protective approach. And that still remains to be determined.

On the forefront of this is thinking about if you can predict the microbiome of an infant and when it shifts, what then can we do at the bedside to be able to prevent this devastating disease?

A lot of people have looked at stool biomarkers because we know that when a baby has NEC, one of the signs that we see is that there's blood in the stool. And so again, microbiome, we have this dysbiosis or these shifts in bacteria that precede a diagnosis of NEC sometimes as early as a week prior. Other stool biomarkers include calprotectin, so this is an inflammatory marker that's really not specific for NEC, but a lot of us like to hang our hat on it a little bit and we check it in mouse models and all kinds of things like that. But ultimately, it's not what we need at the bedside.

Some newer biomarkers include what's called volatile organic compounds or VOCs and this is, I don't know if you've ever heard a nurse say to you, "We think that this baby has NEC because they smell different?" These volatile organic compounds are what we call a smell print that you can put in a baby's incubator, for example, and it's an electronic nose that can smell out if a baby has NEC vs sepsis, so more on that in the future but something to watch out for.

There's a group at Tulane that we collaborated with and Sun Yong Kim's group and in the stool, their team found that there's an intestinal alkaline phosphatase, like an elevated protein, in the stool associated with low enzyme activity that can predict NEC onset. More on that to come.

Shifting from the stool into the urine biomarkers, there's a lot of different urine biomarkers and we have done some work, but this is going back now 10 years. Some investigators had found that urine intestinal fatty acid binding protein, which is a marker of mucosal damage, is higher 3 to 7 days prior to NEC. What would we do if we knew a baby was going to have NEC 7 days prior? I'm not sure, and the jury is still out on that, but it did seem promising at the time.

Urine serum amyloid A is higher in complicated NEC and then another marker, prostaglandin E2 major urinary metabolite, is associated with the length of necrotic intestine. Something like this is too late for us and we want to be able to predict the disease. All of these studies, and there's several others that I don't have time to mention, but all of these studies really are prohibitive because they don't have a lot of samples in their studies or a lot of patients that are recruited.

Everyone asks me, "Well why don't we have a biomarker for NEC, Misty?" And I'll say it's hard work, and when I was a fellow I started collecting these samples, I tried to get other people to join me on this bandwagon and it's really difficult. What I'm going to do is talk a bit about our NEC Biorepository and that's how we're working hard for it.

One of the issues in the field has been that there's not a lot of NEC at one given center and collecting these samples is difficult because, again, it requires a 24/7 operation and a lot of people aren't that dedicated to roll out of bed at 3:00 in the morning and pick up an intestinal sample from the operating room. When I started biobanking, I really wanted to develop a biorepository with a lot of samples from a lot of babies and, for me, it started at 1 center because, again, I was a fellow at the time.

What is a biorepository? So, it collects, processes, stores and distributes biospecimens to support investigation. Now, when I was a fellow, I wasn't able to do a multicenter study, but I wanted to start somewhere so I started at our center. The purpose is to maintain these specimens, obtain them with a great manual of operations and standard operating procedures and really obtain that clinical info for research. One of the barriers has been can we take the specimen and see what exactly is happening to the baby at that given day? Is it that they

are having feeding intolerance? Did they receive a new medication? Did somebody add something to their feedings, sodium chloride for example or other things that can change the intestinal environment? Our job really for the biorepository is to assure the quality and then accessibility and distribution of the samples and so I'll talk more about that.

In 2017, I partnered with the NEC Society to really help advocate and bring in other centers, other than just my friends and colleagues that were on this mission with me, to be able to really start collecting samples and digging into finding a biomarker for NEC. The NEC Society was founded by Jennifer Canvasser, for those of you that haven't heard about it, and they're really our partners in pushing this NEC biorepository forward. Over the last several years, we now have 8 research centers enrolling with over 843 babies and this is updated as of last month, but you can see here all the different centers that are involved and our goal really is to obtain funding for everyone to be able to have these 24/7 research operations and really accelerate the research for this devastating disease.

Here's the map of the biomarker repository sites, and you can see they're all across the United States. We certainly can add more sites and so I'm happy to talk to anyone at the break if they're interested. And to talk a little bit about the administrative structure, so one of the things that I hear about when it comes to collecting samples is that people will say, well it's difficult, I don't want to do the IRB. Don't worry, I've done all the IRBs for you and there is a single IRB for all member sites that are overseen by my center and that usually helps other IRBs feel good about being able to join. All the IRB protocols are standardized and include sharing because that's the nature and the spirit of the biorepository, so they're shared between sites, all the consents are standardized and allow for sharing within the biorepository, but even with other collaborators as well. Then we train and provide support for the research staff at each site so that the PI doesn't have to necessarily train their research coordinator about how to talk to a family that has an infant that's dying from NEC for example. Then we have material transfer agreements and data use agreements between each site to facilitate sharing of data and the samples.

We also provide oversight and training, of course, at onboarding. We have standard operating procedures and a manual of operations, and a shared REDCap database, and we collect all the clinical data from these babies. What are they eating that day that they got that sample and did they have a blood transfusion or a platelet transfusion or any type of exposure actually? And we're looking at the various exposures and how they impact the samples. Are these samples being consistently processed and ensuring that there's proper storage and reagents? And I can't tell you, on the basic science guide from the lab, just how important that is. We actually ship reagents to some of the sites that don't have basic science labs to ensure that they are mixed properly and are stored properly. Then any troubleshooting, let's say you have a surgeon or a surgical nurse or something that won't give you that specimen, we work with the teams to be able to overcome some of those barriers that I personally had to overcome at different centers as well.

What samples do we collect? That's what everybody wants to know. We do collect almost everything. We collect blood at the time of enrollment. We collect, at the time of NEC, also blood. But we obtain all the discarded blood left over in the clinical lab. We partnered with them and they're able to collect everything that's left over and you would be shocked how much blood is just discarded and we use it for our purposes. Then urine, stool, and gastric aspirate, and breast milk, we collect up to 3 times a week and then saliva, we collect once for genomic DNA and then any time there's intestine that's resected for NEC or any indication, 24/7, we collect that as well.

People ask a lot about tissue procurement and so what is a control for a baby that had NEC in terms of their intestinal resection? Examples include a stoma closure, intestinal atresias, spontaneous intestinal perfs, strictures, literally any reason that a baby has an exploratory laparotomy with a resection. When we think about what is non-NEC, they're not control samples because obviously we don't have babies getting colonoscopies, for example, but it's the best that we have and the ability to have an adequate comparison.

We work with the teams to talk about detailed procedures for a 24/7/365 tissue procurement. How to process and storage and we really have tried to streamline those efforts to make it easy for everyone. Then I talked earlier about funding, so in terms of biorepository maintenance, we are not going to stop doing this until we have biomarkers for all of us at the bedside. How do we keep the funding going is an ongoing concern. Again, we partnered with the NEC Society. They do give funding for centers to start up and get things going. They have generously awarded $42,500 to all of the sites that have partnered with us. Then what we do is we work with each center to then obtain their own either internal funding or NIH or foundation funding and we've been incredibly successful in that regard.

One of these awards that we have received a couple of years ago and then just got a second round awarded is in partnership again with the NEC Society, is the Chan Zuckerberg Initiative award for patient-partnered collaboration and so they've given a total of $2 million. So they give $1 million at each round and so it's $1.6 million for science and then $400,000 for the NEC Society for capacity development. The goal really of this work is to find these NEC phenotypes, again at the different gestational

ages, using multiomics approaches and all of this is possible because of the biorepository that we've developed.

Shifting gears to talk about some of the pilot studies that we have done to obtain some of this work and really looking at those different phenotypes and digging in using multiomics approaches. These are some busy graphs, but what I want you to see is that we looked at some of the intestinal samples. On the colon, the colon is on the left and the ileum is on the right and you can see, along the bottom here, the NEC samples compared to the control or the non-NEC samples. What I would like you to appreciate is the dark blue over here in the patients with NEC compared to the controls. What this told is that there's DNA, what we call hypermethylation or elevated methylation globally across the genome and this was one of those markers for NEC. Now, this is at the time of tissue resection, so we all know if a baby's having surgery, it's not necessarily a biomarker if you can just tell us the difference between inflamed and noninflamed tissue.

We wanted to look in a small cohort, can we identify this in the stool and what does that look like? This is a pilot project that we did in a case control study where we were looking at patients that had already been diagnosed with NEC and their stools. You can see this dark blue DNA hypermethylation that's present in infants with NEC. Now, what we have, we're currently R01 funded to look at is a large prospective study across several sites to see is this happening at every gestational age and is this something that we can develop a rapid test for at the bedside to be able to bring it to us all.

We get a lot of blood and so we wanted to look at all this blood that we are taking from our babies, are we able to look at the different proteins in it and can we find something different? We did this study that looked at over 3,000 protein markers in the serum of babies with and without NEC and found just 11 serum proteins actually that were significantly different between infants with NEC and controls. When you think about what are the best ways in which we can diagnose this disease, it may or may not be the serum or it could be a collection of serum, urine and stool. But there are only 11 and a lot of these are some chemokines and other things. We thought that was interesting and I wanted to share that with you.

We looked at, in the serum of these babies, we looked at age-matched controls and then self-matched controls. When we talk about self-matched controls, that's because we're collecting samples prospectively from babies that don't have NEC because we can't tell you which babies are going to get NEC, but the self-matched controls will be a sample from a baby that didn't have NEC compared to the time that they did. There were definitely patterns of different serum protein abundance between these babies and we're digging into this further in a larger cohort.

We recently looked at urine and again did proteomics and what I want you to see is you can see there's definitely a larger difference in terms of urine as a potential fluid in which we can diagnose NEC compared to the serum. This is something exciting that is not published yet, but hopefully will be soon. More to come.

What we found just to distill that down is that there's 2 protein panels that we're really interested in and, on the left here, these are REG1B and then REG3A and DEFA5. These 3 are some antimicrobial peptide-type proteins and so we're digging into that a little bit further, but definitely have these urine proteins panels of interest.

We're doing a lot of exciting work and so we would love to partner with anyone who's interested in becoming a NEC biorepository center. In order to do that, please email me and we'll set up a meeting to discuss with your team and our team and how the biorepository works, discuss the infrastructure in more detail and the data collection that we require in order to be able to say some exposure has been impacted these babies and we'll give you more information that you need and then we add you as a site again with a single IRB, our shared database, so you don't have to set up any of that. We'll get those going for you.

In conclusion, the complexity of the underlying pathophysiology of NEC has impeded progress for decades and discovering biomarkers and preventative approaches and effective therapies really requires a collaborative approach and so we want to partner with you on this. We all really need to work together to build a world without necrotizing enterocolitis.

To complete this course and claim credit, click here.

ANNENBERG CENTER FOR HEALTH SCIENCES AT EISENHOWER

Imparting knowledge. Improving patient care.

This activity is supported by an educational grant from **Mead Johnson Nutrition**.