Part 2: Pompe Disease Newborn Screening Methodology

Operator:

In a listen-only mode. Today's conference is being recorded. If you have any objections you may disconnect at this time. During the question and answer session, which will take place at the end of the presentation, please press *1 on your touch-tone phone. At this time I'd like to turn the meeting over to Ms. Patricia Hunt.

Patricia Hunt:

Hello, everyone. My name is Patricia Hunt, and I supervise the Newborn Metabolic Screening Laboratory in Texas. And I'm also a member of the APHL Newborn Screening Quality Assurance and Quality Control Subcommittee. I'm pleased to welcome you today to participate in the second part of a two-part webinar series titled Pompe Disease 101, Clinical Aspects and Screening Methods. Today's webinar will focus on review of the current newborn screening methods for Pompe disease, states' experiences with implementing the methods, and quality assurance materials available from CDC. Today's first presentation is an overview of available screening methods for Pompe disease presented by Dr. Dietrich Matern. Dr. Matern is a codirector of the Mayo Clinic Biochemical Genetics Laboratory in Rochester, Minnesota. The following presentation will be the Missouri experience

presented by Patrick Hopkins. Patrick is the chief of the Missouri Newborn Screening Lab at the Missouri Public Health Laboratory in Jefferson County, Missouri, and is also the chair of the Newborn Screening Quality Assurance Subcommittee. The next presentation will be the New York experience presented by Dr. Joseph Orsini. Dr. Orsini is the director of operations with the New York State Newborn Screening Program. And then followed by Dr. George Dizikes who will be presenting the Illinois experience. Dr. Dizikes is the CLIA director of the Illinois Department of Public Health and is also the director of the Illinois Newborn Screening Laboratory. And the final presentation will be given by Dr. Hui Zhou and Dr. Joanne Mei from Centers for Disease Control and Prevention. Their talk will be proficiency testing materials for Pompe disease. Our question and answer session will begin immediately following the last speaker. The operator will provide instructions on how to submit your questions once again. And you will also be able to submit questions online throughout the presentation. The questions submitted online will be answered during the Q&A session at the end. As a reminder, the webinar is being recorded for archive today. And a link will be sent out for the archived presentation within the next month.

Dr. Dietrich Matern:

(Section excluded for proprietary purposes)

I pass it on now to Patrick Hopkins from Missouri to talk to us about the newborn screening experience there.

Patrick Hopkins:

Thank you, Dr. Matern. And I'm delighted to be able to share with you all Missouri's experience with our full population pilot screening for Pompe. Just recently in January we completed one full year of testing and have been able to challenge our entire newborn screening system from screening through follow-up, diagnosis, and treatment of Pompe. So on my first slide, we are utilizing digital microfluidics and I'll give you a little background information here. We received a legislative mandate for LSD screening. Missouri's annual birth rate is around 78,000. But we have about 91,000 samples per year with the repeat screens. We chose this method due to cost, space and time constraints. And we are currently conducting a four-plex Pompe, Gaucher, Fabry and MPS-I. And New York is testing Krabbe for us. We conducted extensive validations and prepilot in 2012, and that's when we formulated our startup cutoff. And then we started our full population pilot screening January 11th in 2013 after a full IRB review and approval. Next slide, please.

This slide shows a picture of two workstations. A workstation has one PC and four platforms and so we have a total of eight platforms. And with these we can run two runs a day and up to 608 samples per day. We were able to use this sidewall bench that was currently being underutilized and didn't have to change anything or do anything disruptive in our lab to adopt this method. The redundancy of having all these platforms is reassuring. If you're familiar with a newborn screening lab, we have to have that redundancy, though we've only had one platform break down in one and a half years. Next slide, please.

This slide shows an open platform with the cartridge sitting on the platform. And that's where you plate the samples right on the platform. And to the right is a zoom-in picture of the 48-well sample cartridge. And so the wells on the right side include the stop buffers and the four substrates. And then the lower wells include the four calibrators, four controls, two blanks and 38 dried blood spot samples. Next slide, please.

This is the enzyme reaction in the digital microfluidics method where you have the substrate and then the enzyme that's extracted from the dried blood spot cleaves off a 4-MU component from the substrate. When that's cleaved off you get the product, the free 4-MU product that fluoresces, and glucose. So

low fluorescence means low enzyme activity. So we are looking for low values in this assay just like with galactosemia GALT assay. Next slide, please.

This slide shows the workflow for our testing. The dried blood spots are punched into regular 96-well plate which takes about 15, 30 minutes, depending on how many samples we get. And then there's an extraction phase where that takes 30 minutes on a rotator at room temperature. And then loading. We transfer an aliquot to load into the cartridges, which takes about five minutes per cartridge. We have some staff that can do it in three minutes. And then you close the lid and the platform runs for two and a half hours to get the results. Next slide, please.

Each cartridge has four calibrators. And this is a screenshot from the workstation PC. And so for each cartridge we run a base level, low level, medium, and high level calibrator and get a calibration curve that is applied to all the samples on that cartridge. Next slide, please.

This is a screenshot of a quality control monitor that's provided. We run two low QC spots and two medium QC spots on each cartridge. And the low QC is in the affected range,

positive range. The QC medium is in the borderline to normal range. So those are two good levels to run QCs on every day and on every plate. So the software allows you to chart these QC samples on any and all runs for that morning run or the day's run, the week's run, or the whole reagent lot for several weeks. You can monitor all this. Next slide, please.

This is a screenshot of the results screen. This is what this looks like. We utilize two cutoffs. We have the instrument cutoff, which flags in yellow if that's breached. Or the referral cutoff, which is even lower, which flags in red if that is breached. The instrument cutoff is hopefully our fail-safe cutoff to compensate for imprecision in the blood spots and prevent a true positive from sneaking by us. And then we repunch anything that breaches the instrument cutoff or the referral cutoff and retest that in duplicate. And if the average of the three runs remains below the referral cutoff, then we refer it, as long as everything else in our criteria applies. We also look at the other levels of LSDs that we are screening for. Next slide, please.

So this slide shows the 10 confirmed Pompe genotypes that we have detected in the first year. So in the first full year we detected and referred 33 positive Pompe screens. And these 10

confirmed out with Pompe genotypes. This does not include pseudodeficiencies. The y-axis is the enzyme activity. The green line across the middle is the newborn median or the 50th percentile. And then you can see the yellow line is instrument cutoff. And the red line is the referral cutoff. And the 10 cases across the bottom, the first three are the three infantile Pompe that we obtained in the first year. And these three are all on enzyme replacement therapy. One was nonclassical infantile and two were classical. And we found out last week that the difference with that is for the nonclassical it just means they don't have the cardiac involvement right at birth. And then the next set, the Pompe late onset, we have four of those. And then we have three cases that are genotypes of unknown onset or unknown significance. And so typically what these are, they confirm with low enzyme at the confirmatory lab. And they typically have one known pathogenic mutation along with another one that is not well described. And so it could be possibly benign but it's not for sure. And so these kids have to be followed long term. Next slide, please.

So for our first year's findings we are very pleased with the digital microfluidics methodology. We really like the way it's what I call newborn screening laboratory-friendly. It's very easy to cross-train staff to do. It's working well for us. And

I do like the redundancy of the platforms. In case one is temporarily down there'd be seven left. But I would prefer to be able to have the cartridges complement the 96-well microtiter plates that we punch spots into for all of our other assays. I'm sure that's in future next generation plans for that platform. Like I said, we did have 33 positive Pompes referred in the first year, and with the 10 Pompe genotypes we found 6 others confirmed out to be Pompe pseudodeficiencies, 6 confirmed to be Pompe carriers, and there were 8 false positives, although some of those false positives could actually be carriers. But if the enzyme level confirms in the normal range albeit even low normal, then DNA is not conducted. And then there's three still pending in the first full year. Our positive predictive value is 30% and like Dr. Matern said this does not include the pseudodeficiencies as positive in that positive predictive value, only those 10 that are being continually followed and treated. The false positive rate is 0.026%. Our detection rate so far for infantile onset was 1 in 26,000 and late onset 1 in 19,500. But as you all know, we really need three to five years of data before you can really zero in on what the incidence is on some of these disorders.

The next slide gives other important findings that we've found out. Enzyme activities do drop slightly during the first two

weeks of age and then stabilize after 14 days of age. Now whether this is due to a change in the hematocrit or leukocyte count I'm not sure. But we will need age-related cutoffs for older babies for our repeat screens. We found that premature babies can have altered enzyme levels. With Pompe it seems as though the premature babies have lower levels and could prompt false positives. So you'll need repeat screens. We automatically have repeat screens in Missouri on premature babies as I think most states do. And so we'll make sure we get repeat screens on those unless we decide to override and refer it. We found that multiplexing with other enzyme assay greatly helps assess quality of the sample and risk for referral. And we have seen some seasonal variation observed of enzyme activities, similar to the GALT and biotinidase assay in that more carriers and pseudodeficiencies could be detected during high heat and humidity months. But that seems to be sporadic and in subpopulations. Next slide, please.

This is a picture of Gavin, who was the baby we detected on the second day of our pilot, and I believe is the first baby with infantile Pompe to be detected through routine newborn screening in the United States. And he is doing very well on the enzyme replacement therapy and just turned one in January and he's

walking and meeting all his normal milestones. The other two infants on ERT are doing very well also.

And then finally I'd like to thank all the following people on the acknowledgment slide. It really does take a village to start a new screening disorder. So thanks to all these people. And with that I would like to introduce the next speaker, Dr. Joseph Orsini, who is the New York Newborn Screening Program manager, to give us the New York experience.

Dr. Joseph Orsini:

Hello. Thank you, Patrick. I'm going to have a slightly different approach to the slides. And just on the first slide you'll see a reiteration of last week's where you've got glycogen being in the presence of enzyme going to glucose. And so this is pretty much the principle of the method. So we'll be going to let you know what I'm using in our state. We've had varied method population studies we've performed here. We started using Michael Gelb's enzyme assay that was described by Dieter earlier. And the reason we started with that and have been continuing to work with it, it works really well, number one. And number two, we had no choice because of Krabbe disease. This was the only method that was available at the time when we started, which is approximately nine years ago.

We've since modified the method working with other people to develop assays that will allow for -- basically simplified the original Gelb method and made it so you can run an assay similar to what Dieter reported on earlier with less having to make solutions. So one of the advantages of this right now is we can run one solution and run five enzymes with a second solution, add the sixth enzyme, so it's fairly simple from that perspective. My understanding is as Dieter pointed out that there's a universal enzyme buffer solution being worked on by Mike Gelb and his coworkers that'll allow for simplifying the method yet further from what I'm going to describe. We are currently performing a consented pilot study with Dr. Melissa Wasserstein and we're screening for Pompe, Fabry, Gaucher and Niemann-Pick disease. That assay is similar to what Dieter described in that we could add ALD adrenoleukodystrophy to it with some tweaking as well as MPS-I and Krabbe disease of course. The methods were validated and approved using New York State clinical lab method guidelines and require all the kind of things you'd be used to with interday precision, cross-day precision, accuracy, linearity, etc.

So the basic principle. The method is starting with this thing that's labeled GAA-S, which is the synthetic substrate that has a glucose unit that then in the presence of dried blood spot

with working enzyme will form an enzymatic product labeled GAA-P, with a mass-to-charge ratio of 498. Along with the substrate at the beginning of the assay internal standard is added to the dried blood spot and quantitation is based on a ratio of internal standard to the enzymatic product.

So our method, similar to what Dieter had put up, a five-plex method now actually moved to six-plex or seven-plex if you count ALD. And as I mentioned, you start with two plates for our assay. If you want to do Pompe you would just start with plate one and use a Pompe solution. We incubate for 19 hours. That's primarily due to the enzyme for Krabbe being fairly slow-acting. With Pompe you could I'm sure get away with less time. But for us if it's going to be longer than a two-, three-hour timeframe it just makes sense to let it go overnight so that you can actually come and start the rest of the process. The next step in the process is to quench the enzyme reaction with ethyl acetate, and then perform a liquid/liquid extraction. You take a portion of that top layer of the liquid/liquid extraction, centrifuge, perform a solid phase extraction, which I will say is optional, if you're running just Pompe or if you're running fewer enzymes, you can make solutions that start with fewer materials, and so the solid phase is -- for us we found it useful. It made for really nice clean extracts on a mass spec,

which made for a little less maintenance, or actually a lot less maintenance on the instrumentation. But those issues are being also worked on by Mike Gelb now to eliminate solid phase extraction. We do use it. We've got it automated with liquid handling equipment. And we can set up to run solid phase in five minutes per plate and walkaway technology. So it's fairly simple. And I'd be happy to show people that technology if you'd like to see it or come by sometime.

So this is just the steps again. I will skip down to the last step, which is calculating activity for each sample. We use the daily mean activity which allows for some fluctuation in winter or variation maybe from assay solution to solution. There is some slight differences. And it makes for having to worry about cutoffs is all relative to the daily population. For us that is simple because we have such a large number of samples. That's the one thing we can count on to be constant is really the daily mean. Or you could use the median if you have a smaller population.

These are some of the statistics that we have. I'm not going to go over all these. There's lots of numbers here. But we've run multiple enzyme assays. We've run our four plus one, which I just described. We're running ALD with a triplex assay. And

all three at the top you'll see the means of 16.7, 15.2, 15.4, all fairly similar. And minimum enzyme activities being fairly similar.

And if you look at the next line it says 20% and 15%. Those are percent of the daily means. You'll see similar numbers coming out. Out of 5,000 in the two four plus one studies we were getting 6 under 20% and 3 under 15%. Our Pompe cutoff is currently 15%. That's very conservative from my perspective, because if you look at the table in the middle of the slide, you'll see all the positive controls we've been running and their percent of daily means are well below 10% on all the positive controls we've tested. But in our experience we've found that positive controls from older children aren't always as reliable as those from babies. And actually some of these are from babies. And they were quite low. And that was from some samples that were sent to us from Patrick Hopkins that he identified through his screening. Thank you for that, Patrick. The last table is just a comparison of some methods that were in the literature. And we were all very similar with population means, mins and maxes. And one of the things you'll see in the bottom right-hand corner is the Austria lab and Taiwan lab had percent of means when I compared that was closer to 15%. So

that was one of the reasons we chose 15% as our cutoff, although all of our evidence says it could be lower.

So coming back to how do you choose an assay. And I think Patrick touched on why he picked his assay. The primary reason we picked what we're using here was that we had to run it with Krabbe but it's become very -- I really do like the flexibility of the lab. And for those of you who don't know, we recently added adrenoleukodystrophy and as Dieter had pointed out, you can parallel process plates for adrenoleukodystrophy and then run them on the same exact plate on a mass spec. So there's no additional equipment needed to run ALD in our laboratory here at New York State. We just added it with Krabbe disease, which made it relatively cheap. So a big thing to consider is if you're going to run Pompe disease what other disorders you may need to run in the future. And if you have to run Krabbe disease then like some of the states that are out there now, then mass spec really is still the only option. Although my understanding is that the Advanced Liquid Logic platform people are working very hard to get together an assay that'll work on the platform Patrick is using as well.

Budget and space. With the mass spec technology right now at least once you purchase the equipment, the materials for the

assay are actually relatively cheap. And I think if you look at the cost over 5 or 10 years, depending on what happens with the kit in the future and the costs for that, the costs become fairly comparable. Just a matter of if you spend the money up front or if you want to spend more as you go with purchasing kits. Also staff capabilities will be a consideration. I already mentioned if you have to run Krabbe or Niemann-Pick those currently can only be done by mass spec. And then the not so obvious considerations I already mentioned. Will you run ALD? Because you could run ALD with this kit. That depends also on whether you're using derivatized or underivatized kits for normal amino acid and acylcarnitine analysis. If you're going to run ALD you can actually run it in underivatized kits. So those are some considerations. And if you needed more information I'd be glad to go over some of that with you as well in a private conversation.

So conclusions. The validations for the methods all have gone smoothly. They have very good sensitivity by the method and comparable to what Dieter has mentioned. If we had to do it again we'd likely choose the same because there's no option as far as Krabbe. I would be in the situation where if we had to choose and if we were only running for Pompe it would be a little bit tougher decision. But given the fact that we're

running adrenoleukodystrophy and possibly may run MPS-I in the future, I do like the ability to multiplex ALD with the LSDs.

And then one thing to consider with this is with the multiplex enzyme assays the materials, the substrate and internal standards, are provided by the CDC, which is nice. But they're in vials. And it actually gets fairly complex if you're trying to prepare large volumes for a state such a size as ours. It's fairly cumbersome, and that's something that would need to be addressed for larger states anyway to make things simple. There's a lot of vial preparations. Even though in the end you only end up with two solutions. Or if you're just doing Pompe it would be a moot point. We'd only have one set of vials. But if you start multiplexing you have many sets of vials that need to be combined to make this assay work.

And then the last slide is just my acknowledgment slide for folks here at the state that have helped. Monica Martin and Amanda Showers. Of course working with Dr. Melissa Wasserstein and Nicole Kelly at Mount Sinai and Bob and Hui for providing us with all the CDC controls. Dieter for sharing his ALD perturbation on the method and Coleman. And Mike Gelb for all his insight as time has gone through the years of working with this assay. So thank you very much. With that I'd like to pass

the talk on to George Dizikes from the Illinois Department of Health.

Dr. George Dizikes:

Thank you, Joe. And I want to thank APHL and the organizers for this opportunity to present Illinois's experiences developing a newborn screening assay for Pompe disease and other lysosomal storage disorders. Before beginning I'd like to acknowledge my coworkers at the Illinois Department of Public Health Newborn Screening Laboratory at Chicago and others who helped supply technical and material assistance in developing this assay, including Dr. Burton, who supplied known positive blood spots, Drs. Gelb and Orsini for technical assistance, and the CDC Newborn Screening Molecular and Biology Branch and PerkinElmer Corporation for technical and material assistance.

In 2007 the Illinois General Assembly passed legislation mandating testing for five lysosomal storage disorders including Pompe. The others are Krabbe, Gaucher, Fabry and Niemann-Pick A and B. In 2010 a pilot study was begun involving two large birthing hospitals in Chicago. Testing for Pompe, Gaucher and Fabry was performed on a microfluidics platform similar to the one later adopted by Missouri. In all over 8,000 specimens were tested and two presented with reduced GAA activities. These two

cases were later determined to be normal by second tier tests. In 2011 the legislative mandate was expanded to seven LSDs by the addition of MPS-I and MPS-II. Added to this legislation were the following provisions that needed to be met before screening could begin. A method for testing needed to be available that was either FDA-cleared or had been validated under CLIA. There needed to be quality control and proficiency testing material available. Appropriate equipment and space needed to be available to support high volume screening. And there needed to be adequate funding to both develop and maintain the testing.

State of Illinois screens more than 170,000 newborns a year. A decision was made in 2011 to switch from the microfluidics platform to tandem mass spectrometry based on the throughput of the microfluidics platform and the fact that fluorescent substrates were not available for two of the disorders, which has already been mentioned, Krabbe and Niemann-Pick A and B. Also developments in multiplex tandem mass spectrometry for LSDs had progressed from the time that the original decision had been made on testing platforms. And these new developments promised to provide adequate testing throughput for more disorders and with less staff than using microfluidics. Statewide testing for

Pompe and five other LSDs is scheduled to begin July 1st of this year.

The assay that we ultimately implemented is a modification of the method developed at the University of Washington in the laboratory of Michael Gelb and is based on work published by Duffey in 2010 and Spacil in 2011. The assay utilizes a single three-millimeter dried blood spot punch and a single buffer with detergent to accommodate both hydrophobic and hydrophilic substrate. A short three-hour incubation time is used to optimize workflow. And incubation products are separated by ultra high performance liquid chromatography or UPLC. To reduce cost, complexity, and instrument maintenance, a single column is used with valving to waste before injection to remove low molecular weight materials and after injection to eliminate detergent and phospholipids. There is no solid phase or liquid/liquid extraction. Injection cycle takes two and a half minutes which permits over 500 injections per instrument per day. And elimination of potential contaminants has extended the time between preventive maintenance to over 10,000 injections.

This slide shows the Waters Acquity TQD instrument. The refrigerated plate holder on the left. The UPLC unit is on top of the center stack with the autosampler below and the various

valves and pumps contained in the unit below that. The mass spectrometer is on the right. We have four of these units in the lab.

The next slide shows the elution pattern for the six internal standard product pairs in the multiplex assay. Of note is the fact that each pair has the same retention time so that both species experience the same degree of ion suppression. The pair for GAA elute at about 0.6 minutes. To accomplish this paired separation for the hydrophobic species the internal standards for the reactions associated with Gaucher, Fabry and Niemann-Pick A and B were labeled with d7 rather than d5 as suggested by Michael Gelb. When separated by mass differences the products and internal standards are detected in 12 MRM channels.

This slide shows the linearity of the assay for GAA using QC low and QC medium filter. These filters are prepared from pooled cord or adult blood. The white cells are removed and then added back to approximately 5% cellularity for the low control and 50% for the medium control. The high control, which is not shown, has 100% cellularity. The low control has activity around what a positive case would exhibit. That is about 5% to 10% of normal. And as can be seen, even after three hours there is a linear accumulation of product.

Once the details of the assay had been settled on including buffer composition, design of the internal standards, concentration of the standards and substrates, incubation time, UPLC conditions and valving, and settings for the mass spec, the method was validated in the following ways.

The different levels of control material, low, medium and high, were run on various days on the same instrument. And comparisons were also made between the four instruments we're using to determine precision and accuracy. Numerous deidentified blood spots were also tested in the same way, punching a single specimen multiple times, and making multiple plates from a set of specimens for analysis across the instrument. We also participate in the CDC's proficiency testing program for Pompe and Krabbe. And a number of blood spots from confirmed positive cases had been obtained and tested.

The effects of sex, low birth weight, and age at the time of specimen collection have also been evaluated, as have the effects of storage conditions on the dried blood spots and potentially interfering substances such as detergents. Cutoff values are being estimated based on the distribution of

activities in a largely normal population and by referencing the activities in known positive specimens. A specimen exchange with a qualified testing laboratory is also being conducted to demonstrate comparability of results.

This slide shows assay results for over 10,000 randomly selected deidentified residual dried blood spots. Confirmed Pompe cases, CDC positive Pompe proficiency test materials, and the low, medium and high control dried blood spots. The horizontal lines indicate the mean activities for each group. And the confirmed cases, positive PTs and low control have about the same activity. Those are in the second, third and fourth positions.

These data points are statistically analyzed here. Of particular note is the greater than 12-fold difference between the mean GAA activity for random deidentified dried blood spots of 7.45 micromoles per liter per hour and the dried blood spots from confirmed Pompe cases which is 0.58. Also up to this point there does not seem to be overlap between negative and positive specimens. The minimum GAA activity for the 10,000 deidentified dried blood spots was 1.12 micromoles per liter per hour while the maximum activity for the confirmed Pompe cases was 0.69. And for positive CDC Pompe PTs it was 0.94.

In addition, the low, medium and high controls show good linearity, with the low control exhibiting GAA activity approximating that of confirmed cases. Also analyses of the control dried blood spots indicate good reproducibility as shown by their standard deviations which are in the right column of the upper panel. Good reproducibility for the assay is also supported by interday and intraday testing of multiply punched random deidentified residual dried blood spots which is not shown here.

So what have we learned? Many different individuals with a wide range of skills need to work together to successfully develop a complex high throughput statistical analytical assay. The process will take longer than initially anticipated and regular interactions and good communications are vital. Compared to microfluidics the MS/MS platform permits an expanded test menu and multiplexing with a single injection. And with regard to using this MS/MS platform for a dedicated GAA assay as opposed to the multiplex assay, synthesis of the d7 internal standards would not be required. And a simpler reaction buffer could be utilized which does not require detergent. Operation and valving of the UPLC column could also be made more efficient leading to shorter times between injections and greater

elimination of contaminants resulting in more injections between scheduled maintenance and a lower cost per specimen.

There are many challenges in adapting a research procedure to a high throughput newborn screening assay involving analytical, personnel, physical plant, and IT issues. FDA-cleared assays would be vastly preferable in terms of development and validation of the assay. And if at all possible if there is a push to mandate testing in one's state, it would be highly advised to have legislation or administrative rules written to permit adequate preparation and milestones before testing needs to commence. I'd just like to add that a version of what I've presented here can be found on the NewSTEPs Web site under technical assistance and model practices. Thank you. And now I'd like to turn the webinar over to Drs. Hui Zhou and Joanne Mei who will be speaking about proficiency testing materials for Pompe disease.

Dr. Hui Zhou:

Thank you, George. And this is Hui. I will talk about how we prepare the dried blood spot PT materials. The PT materials should be disease-specific. They can be obtained directly from the blood unit of affected patients. However, the availability is very limited. An alternative approach is to use EBV-

transformed lymphoblast cells derived from such patients. We purchase the cells from a commercial cell bank and maintain them in our laboratory. The cells are added to human leukocyte-reduced blood at the concentration of 30 million to 50 million cells per ml blood.

To prepare the dried blood spots the hematocrit level is adjusted to 50%. The blood is then spotted on filter paper, dried and spotted and stored frozen with desiccant in sealed bags. The DBS samples are evaluated for six enzyme activities using our reference method. Six individual enzyme reactions in optimized matrices followed by six-plex flow injection analysis and mass spec.

The DBS prepared using transformed cells from a healthy individual shows a normal profile of the six enzymes as is shown on the left side. For Pompe-specific DBS only the Pompe enzyme GAA is deficient while the other five enzymes are normal as you see in the right side. Several newborn screening laboratories have also tested this material using their own methods. All results showed deficiency of GAA and normal levels of other enzyme. This Pompe-specific material has been used by NSQAP for proficiency testing. And next Joanne will describe to you the PT program.

Dr. Joanne Mei:

Thank you, Hui. So the Newborn Screening Quality Assurance Program at CDC operates a pilot proficiency testing program for Pompe and Krabbe diseases. We send out specimens four times per year. And the information that we collect from these samples includes analytical data, the clinical assessment for each specimen, the method used and method-specific cutoffs. We have a one-month turnaround time for reporting data. And we supply summary reports within about two weeks after the data reporting deadline.

In 2013 we sent the same proficiency testing specimens that Hui produced in randomized panels. Specimens one, two and three exhibited normal levels of the enzyme GAA. Specimen four was abnormal for the Pompe enzyme. Three methods were reported to our program. They included flow injection analysis mass spec in the dark bar on the left, LC mass spec in the central striped bar, and digital microfluidics in the last bar in the hashed bars. So you can see that all methods were able to detect the normal levels of the enzyme while the abnormal method was very low for the GAA enzyme.

Sorry for the delay. I'm having a hard time advancing slides. This is a summary of the analytical data that we collected. You can see that the flow injection mass spec and LC mass spec methods had very similar analytical results. And the digital microfluidics method was somewhat higher than the other two. Each method detected low levels of GAA and those levels were below the cutoff reported for each method. And the cutoffs are listed on the far right of this table.

The qualitative results that we collect include within normal limits or no follow-up required or follow-up required. And we use that information to calculate false positive results and negative errors. For 2013 we had no reported false positive or false negative errors on these proficiency testing materials. So each laboratory had a 100% satisfactory result.

So in summary the CDC LSD reference materials can be used for assay development and validation, for all the methods that are currently in use. The EBV-transformed lymphoblast cells that Hui produced are derived from LSD patients. And they provide a sustainable resource for condition-specific reference materials. And NSQAP's pilot PT program for Pompe, the results we have from 2013 indicated no false positive and no false negative results reported for those PT specimens.

To receive materials please contact Dr. Hui Zhou. Her information is there. For PT materials you can contact myself or Hui. And if you need proficiency testing instructions or how to report data to the program you can contact Irene Williams. And that’s it. Thank you for listening to our webinar. And I’ll turn it back over to Patty Hunt.

Patricia Hunt:

Thank you very much. Thank you to all the presenters for their wonderful talk today. I think we’ll wait for the operator to activate the question and answer session.

Operator:

Yes, ma’am. Thank you. We will now begin the question and answer session. If you would like to ask a question through the phone, please press *1. Please unmute your phone and record your name clearly when prompted. Your name is required to introduce your question. If by chance you need to withdraw your question press *2. If you are using the net conference and would like to ask a question through the net conference, select the Q&A menu, enter the question into the type a question for the presenter box, and then select the ask button. One moment, please. I will monitor the phone Q&A.

Patricia Hunt:

While we're waiting for the phones to connect I'll go ahead and ask the first question. It's for Dr. Matern. Did you see any issues with low birth weight infants in any of the methods that you were comparing, Dr. Matern?

Dr. Dietrich Matern:

Yeah, this is Dieter. That's a good question. We have not looked at the birth weights and ages at time of collection to see whether that makes any difference. I noted that in I think Patrick's and George's presentation. So that is certainly something that we will still look at.

Patricia Hunt:

Thank you. The next question is for Patrick Hopkins. And why did you decide to use fixed cutoff versus floating cutoff?

Patrick Hopkins:

Well, that's a great question. Well, we really prefer fixed cutoff in our newborn screening lab if we can do that. We have fixed cutoffs for galactosemia and that enzyme assay. And we really watch that closely and we feel like the cutoffs are

stable enough that we can do that as long as we have a separate cutoff for older babies. So we have a cutoff for less than 14 days of age and greater than 14 days of age. Plus we carefully monitor the quality control of each -- the daily mean and the daily median and the shifts in new reagent lots. And so we can adjust the cutoffs if we have to if there's a change in a reagent lot and we've done that one time with the Fabry cutoff. But the other ones have been pretty stable other than just changes that we made because of the feedback and false positives. So with the seasonal changes in the summertime we think that's more of a subpopulation that's spotty and sporadic and doesn't necessarily affect the whole population of babies. I hope that answers the question.

Patricia Hunt:

Thank you, Patrick. Operator, do we have any questions from the phone line?

Operator:

Yes, ma'am. We have one question in queue. The question comes from Mr. Michael Gelb I believe. His line is now open.

Michael Gelb:

Can you hear me?

Patricia Hunt:

Yes, we can hear you.

Michael Gelb:

Yeah, thank you for all the presentations. I just wanted to make one quick comment. I think false positive rates are important here obviously. And I think it's hard to compare false positive rates. It depends on how high your cutoffs are. It depends on what the second method of analysis is. It depends on what kind of mutations you find, it depends on what kind of disease people you have in your state, depends on the population distribution. I would urge everybody over the next several months to look at one number. And that would be the ratio of mean normal activity for Pompe disease divided by the mean activity for confirmed affected let's say early onset Pompe disease. And I would propose that that ratio should be as high as possible. And that is a very simple statement. My contention would be the higher the better. And the assay with the higher ratio will over time give the less false positive rate. I mean that I believe has to be true wholeheartedly. And I would ask people to look at that number because I think if we get into a comparison of false positive rates between different

places it’s just really hard to get to the truth. And that’s all I need to say. Thank you.

Patricia Hunt:

Thank you very much for your comment. The next question is for Dr. Matern. Well, actually it’s for the rest of the group. It says, “Dr. Matern talked about the second tier analysis including molecular genetics. Are Illinois, Missouri and New York also performing the molecular genetics for second tier analysis?”

Dr. Dietrich Matern:

This is Dieter. So we did not perform molecular as a second tier test. These were deidentified specimens. We used molecular genetics as a confirmatory means to figure out whether the abnormal result was true or not. That’s all I can say about us.

Dr. George Dizikes:

In Illinois the positive screening results would be passed to the short term follow-up program. And then the follow-up protocol I believe for Pompe does involve some DNA analysis. But that would be handled outside the screening lab.

Dr. Joseph Orsini:

This is Joe from New York. Currently we're screening with Mount Sinai. They have a second tier analysis for Pompe and all the other disorders that we're screening for. And our plan was when adopting here too as well to add second tier DNA testing as well.

Patrick Hopkins:

And this is Patrick in Missouri. And we are not going to have second tier DNA testing in the screening lab. Ours is similar to Illinois. A positive screen is referred and the contracted genetic referral centers will confirm the enzyme activity and do the genotyping.

Patricia Hunt:

Thank you. Dr. Matern, can you tell us what is your second tier testing at Mayo?

Dr. Dietrich Matern:

So for Pompe disease as part of the study the second tier test was the method most recently I think published by our colleagues at Duke University. Deeksha Bali was the first author. And that goes back to the original assay by Nestor Chamoles. So

it’s a fluorometric assay that we used as a second tier when one of the first tier assays was abnormal.

Patricia Hunt:

Thank you. All right. The next question is from whom do the labs get the calibrators, controls and internal standard material from.

Dr. George Dizikes:

For Illinois, we purchase the internal standard substrates and control blood spots from PerkinElmer Corporation. And it’s through those purchases that we reagent-lease the instrumentation.

Dr. Joseph Orsini:

For New York, the CDC is providing the internal standard substrate vials that we are currently using.

Patrick Hopkins:

And in Missouri we get all that from Advanced Liquid Logic Illumina Company. Similar to Illinois a reagent rental type scenario. So it includes the controls, reagents, substrates. And then we also utilize CDC’s quality control materials.

Patricia Hunt:

Thank you. Operator, do we have any questions on the phone line?

Operator:

No questions at this time.

Patricia Hunt:

The next question is, are all of the pseudodeficiency mutations for Pompe known.

Dr. Dietrich Matern:

This is Dieter. As we have seen in newborn screening before, probably there are more out there than we know. And so there will be work that needs to be done to figure this out. And unfortunately this can take quite some time to figure out because again the enzyme activity is low and you find basically mutations you haven't seen before. And then you follow the patient and given that there's a lot of late onset Pompe disease out there you might have to wait a long time in order to figure out whether it's pseudodeficient.

Patricia Hunt:

Thank you, Dr. Matern. Operator, any questions from the phone line?

Operator:

Yes, ma'am. Mr. Michael Gelb has returned for a question. I will now open his line.

Michael Gelb:

Everybody can hear me?

Patricia Hunt:

Yes. Go ahead, please.

Michael Gelb:

Yeah. I just wanted to chime in because even though we weren't on the webinar, in Washington State Ron Scott had done a pilot study with 110,000 dried blood spots from the Washington Department of Health Newborn Screening Lab for Pompe disease. And that's published. So we found only 17 samples out of 110,000 for Pompe that were below a conservative cutoff of 15% enzyme activity. And our plan is to go straight to genotyping, which is done off site at University of Washington Children's Hospital. And we don't need a second tier assay. We don't see the point because from the same blood spot without calling back

the baby we get genotyping. And for 17 samples a year, roughly 110,000, we don't see the point of a second tier assay. Thank you.

Patricia Hunt:

Thank you. The next question is for George. And it is what did you do to decrease the incubation time for Krabbe reaction versus the 19-hour incubation that New York uses.

Dr. George Dizikes:

Well, in our hands three-hour incubation seems to provide enough separation at this point. Although we haven't fully evaluated the assay with doing comparisons with the referral lab. But at least as of now we feel three hours is sufficient time for this assay.

Patricia Hunt:

Thank you. I believe that is all the questions we've had today. And Jelili Ojodu will give us some closing remarks.

Jelili Ojodu:

Well, thank you, Patty, and thank you to all of the presenters for the two-part webinar on clinical aspects and screening on Pompe disease 101. Really like to take the opportunity to thank

the presenters. And they took a good amount of time in putting together the presentation that you heard for part one and part two of the Pompe disease webinar. So thank you all.

This particular webinar was cosponsored by the APHL Newborn Screening Quality Assurance Quality Control Subcommittee of the Newborn Screening and Genetics in Public Health Committee. The names of the committee. The names of the committee members are noted on the screen there, Patrick Hopkins being the chair of the subcommittee. And so it's cosponsored by the Newborn Screening Quality Assurance Program at CDC. And we'd really like to thank them also, especially the folks that worked tirelessly on this particular webinar.

Special thanks to Irene Williams and Ruhiyyih Degeberg. All of the activities, the presentations and the logistics of this particular webinar was made possible and successful with their help. And so many thanks to you all.

And then finally I'd be remiss if I didn't plug our upcoming Newborn Screening and Genetic Testing Symposium that's coming up in October, October 27th through the 30th. It's going to be in Anaheim. It's going to follow the themes, the regular themes of a three-and-a-half-to-four-day meeting that we would have every

18 months, the last one being in May of 2013 when we celebrated 50 years of newborn screening, celebrating the past, and preparing for the future. The current theme of the next symposium is Newborn Screening: Reassessing Business as Usual. We will have concurrent sessions on quality assurance and quality control as well as follow-up in newborn screening, whether it's short term and long term follow-up. The call for abstracts for the meeting in question will be out in early March. So sometime next week expect to see announcement for the call for abstracts. For oral abstracts, poster abstracts, roundtable sessions, and other forums, as it relates to the Newborn Screening and Genetic Testing Symposium. We will also be looking forward to hearing from the community on soliciting named awards for the Harry Hannon Award and the George Cunningham Awards as well. And so please look out for that. You will also have the exhibits for the first several days of the meeting as well as the meet the manufacturer's sessions during the symposium. We will continue to update the APHL Web site for the symposium on aphl.org. And so please check that out. Again I'd like to thank the presenters of this particular webinar. Pompe disease has been one of those conditions that has been very timely. The Secretary's Advisory Committee has recommended it. And I know that the Secretary of Health is

considering the next steps on the Secretary's Advisory Committee's recommendation in moving forward.

As it relates to this particular webinar, for anyone who was not able to participate on the first or the second webinar, all of the presentations will be archived on our Web site aphl.org within the next four weeks as Patty had noted earlier. And so if you have any questions about that feel free to connect with any one of us at APHL. All of this would not be possible without the funding and support by the Centers for Disease Control and Prevention, the Newborn Screening and Molecular Biology Branch there that funds the activities of the Quality Assurance and Quality Control Subcommittee. And so many thanks to them. That concludes the webinar. Thank you all for joining us this afternoon. And have a good afternoon.

Operator:

Thank you very much. This does conclude today's conference. All participants may disconnect at this time. Leaders, please stand by for post conference.

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