MPS I: Overview of Available Screening Methods

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Transcript

Hari Patel: Welcome to the third part of the three-part MPS I webinar series on implementation considerations, clinical aspects, and available screening methods. This webinar series is co-sponsored by the APHL Newborn Screening Quality Assurance/Quality Control Subcommittee of the APHL Newborn Screening and Genetics in Public Health Committee and the NewSTEPs New Conditions Workgroup of the NewSTEPs Steering Committee.

Today's one-hour webinar will focus on available screening methods. I'll briefly review today's agenda. First of all, there'll be an overview of available screening methods by Dr. Joe Orsini, followed by his experience with the tandem mass spec MPS I assay. Then another review of the digital microfluidics MPS I assay by Patrick Hopkins. Then, we'll have a presentation on reducing false positives using the CLIR and second tier method by Dr. Piero Rinaldo. Then, finally an overview of CDC quality assurance material by Dr. Francis Lee and Dr. Robert Vogt.

First of all, I'd like to introduce Dr. Joe Orsini. Dr. Joe Orsini is the Deputy Director of the New York State Newborn Screening program and Laboratory Director of the Lysosomal Storage Disease Testing Laboratory. He's a recognized leader in the area of newborn screening for LSDs and is currently collaborating with Dr. Wasserstein at the University Hospital of Albert Einstein College of Medicine on a three-year consented pilot to screen for MPS I and several other LSDs. He's also been invited to lead a national effort in quality assurance and quality control and to develop guidelines for LSD screening. Dr. Joe Orsini also serves as a co-chair of the APHL Newborn Screening Quality Assurance/Quality Control Subcommittee. With that, I'll turn it over to you, Joe.

Dr. Joseph Orsini: Okay. The title of my slide is Newborn Screening for LSDs and with an emphasis on MPS I. The structure you see below is actually the enzyme, which those with the deficiency for MPS I, this is a structure of the enzyme, which is published in 2013. Next slide, please.

Laura Russell: Dr. Orsini, are you there?

Joe Orsini: Yes, I'm here. I didn't see the slide change for a bit. There was a lag.

Laura: Okay

Dr. Joseph Orsini:i: Okay, here's an outline of my talk. The first thing I go over, have an overview of the screening methods. Then, we'll look at the New York assay, New York testing algorithm, some screening data from various state programs with a focus on the New York screening data, and then a summary.

I'd like to say we are a pilot lab for this screening. My experience really comes from screening for other LSDs with Krabbe screening going on for close to 10 years now and Pompe screening for over three. A lot of the lessons we learn through this screening is what gives me the focus of this talk. Next slide, please.

The first step part of the process here is an overview of the assays. I'll just briefly discuss the fluorescent assay as Patrick Hopkins will be going over this but the original fluorescent assay by Chamoles and coworkers was a single enzyme assay based on plate reader. It was piloted in Taiwan who's now using it in MS/MS testing. More recent high-throughput tests, the one that Patrick will be discussing is a digital microfluidics platform developed by babies on the assays known as a SEEKER assay. This is an FDA-approved kit that can screen up to four LSDs. Both of these assays use an umbelliferyl substrate, which fluoresces when the iduronic acid [inaudible 00:04:32] cleave from the molecule. Next slide, please.

The next assays to be developed were HPLC assays in … Illinois is actually one of the first states to implement this testing. This is really following … There's a more sophisticated test that was developed by Gelb and co-workers and can screen up to nine LSDs. The advantage is there's less pre-analytical workup. Once the test is set up, it's easy to add other tests that may require the use of column for separation of analytes. Illinois reports that the HPLC test is working well. They are staying with this test because their plans are that it could be flexible for added test in the future. With proper preventive maintenance, this is working very well for them. They replace columns every 10,000 samples and guard columns every 3,000 samples. Next slide, please.

The assay that I'm going to be focusing on the most, which is the assay we have experience with is the flow injection tandem mass spectrometry assay. Originally, it was an assay that was developed by Gelb and co-workers and folks from the Genzyme provided the analytes. The analytes were provided to the CDC later on. Then, eventually Perkin Elmer, working in conjunction with Mike Gelb, developed these analyte-specific reagents. They're a little simplified the assay. They're using uniform buffers so all the enzymes can run in a single buffer and the structure and inter of the substrate and internal standard are shown in the bottom left corner and very similar in the sense, it's got an iduronic acid that's cleaved and you're using tandem mass spec to quantify enzyme activity. Next slide, please.

Here are the state screening for data for MPS I. This is a slide similar to what Sarah Bradley last week, if you listened to her talk. Roughly, at this point, we've crossed all the States have screened about 800,000 newborns and there have been seven cases detected. It's fairly on track with the incidents that was

expected, although one of the cases in an attenuated case as reported by Kentucky. Next slide, please.

This is the typical assay for those of you who've seen, I've reported before. If we punch a three-millimeter punch, it's a modified version of the assay presented by Gelb. We do an 18-hour incubation. This could be shorter. I thing one thing we've considered is running a shorter enzyme as a first run through and then for samples that require retesting, run with an overnight assay. This would allow for detecting low-enzyme babies in a faster fashion and more timely manner that people are focusing on these days.

Then, there's organic liquid/liquid cleanup and samples are re-dissolved in an MS suitable solvent. We run each test sample, if we calculate the activity per sample and calculate a daily mean activity. We use a percent of daily mean. I think we started this with Krabbe primarily because we were having some lot plot variability. Now, with the lot sizes, this variability goes away but we're still very happy using a percent of mean. I think it becomes very useful when comparing across labs as well as when you're comparing, if you do a multiplex enzyme to get a better feel for a sample quality or potential sample-related issues that can be identified very easily with this, at least through visual eye checking. Next slide, please.

Here's our current IDUA assay. We are running 4-plex with this. We're running four different enzymes. Anytime that IDUA is less than 20%, we retest in duplicate for all four of those enzymes. If they're multiple low enzymes, as on the left side of the slide, we request a repeat. This can be tricky. I'll show you later. Sometimes, it's obvious. If everything is really low, then it's just a poor sample. By that, I mean multiple enzymes below 20%. In enzyme is less than 15% of daily mean after the retesting, then that's a referral. You'll notice we don't have second tier in our current MPS I with second tier DNA. That's something we plan on doing once we go live. The greater 15% will be screened negative. The next slide, please.

This is a summary of New York results. So far, no MPS I cases detected in 31,000 infants and nine infants have been referred. That's obviously a high referral rate. Remember, this is a piloted study. We did purposely set the cutoff at a higher number just to get a better feel for the assay and how things would correlate since these were consented sample testing. The next slide, please.

This slide shows a summary of New York's referrals. What you'll see is, so far, once they've made it to a diagnostic lab where there is full molecular testing, it's just later than what would be done if it were done in our laboratory, were found primarily pseudo deficiencies, so with five of the seven cases being pseudo deficiencies only is the patient not seeing. We're still waiting results on that.

But I'd like to point out that there's one sample in the middle of the slide or the data with the 3.9 IDUA percent, you'll see that one has some fairly low enzyme activities for other enzymes with GAA at 32%, GBA at 34.5%. That would be a

sample that has some suspicious as being, it's really not something that you'd be as paranoid or panicked about with a 3.9%. Again, you'll see when I'm getting to some of my later slides, this will bring this back into focus. An interesting thing I've noticed is a little bit of a correlation between diagnostic lab results and the dried blood spot results. That's something we did not see with the Krabbe testing. Next slide, please.

Just an overview of our plate map and QC. We run a fair number of blanks, as you'll see. Three blanks per plate. We run negative and positive QC. We put a negative QC ahead of a blank. In this case, we're wanting to be sure there's no carry over so it's a measure of instrument carryover per plate. We have multiple blanks on there. That's also important when you're running a test where you're looking for the low enzyme in a sea of high-enzyme activity. We're looking for any kinds of plate sample to sample carryover. This helps with that.

We do average the blanks. We track the daily mean and some people may chose to use median. I'm going to say, I think if you're a smaller lab, you'd chose median but with MPS I, it seems to be a little less of an issue. You'll see some of that in later data.

One thing that helps in tracking both of those is if you look at the daily mean of your samples and compare it to the daily mean of your high-level control, what that will allow you to do is see the seasonal effects because you'll see examples may dip with time in the summers but the QC, of course, not being affected by the environment of shipping will, you won't see that effect. It gives you a better idea of what's going on. Next slide, please.

Okay. This is where I want to describe the feel for the assay. I think what this is going to set up is really why one might want to look at something like using the CLIR database that Piero Rinaldo's going to talk about later. We're going to look at samples with low and high IDUA activity, subpopulation data, which will be low birth weight or low gestation age or older children. Then later, we'll be setting off cutoffs and positive controls. We're looking at how that we've done this sort of thing, at least in the version where we were using cutoffs as we are right now. Let's check the next slide, please.

Out of 32,000 samples, one of the things I'm able to do is just look at all the samples where IDUA is less than 20%. Then, I collate that data and calculated the mean for each enzyme. This is where I think it is useful to have things in percentile because it's very easy for somebody who's mathematically challenged to understand what's going on. If you look at the less than 20% of means on the right side of the table, you'll see that all the enzymes are generally low. If you look at the IDUAs that are greater than 300% of the mean, the enzyme are correspondingly high. This is really why, in a nutshell, CLIR can be very useful because it acts as a way of normalizing the data within the normalized data or across all the enzymes. Okay. The next slide, please.

This slide exemplifies the issue. I grab the samples that were less than 10%. All the yellow ones were samples that were referred. You can see some of them have moderately low other enzymes. There's one with 56, it's the middle yellow one at 56% GALC, GAA's 49%, GLA, 70%. Here's a sample that's just not comparable to, say, the very bottom yellow one where you have a 5% activity, and some samples with means being much higher than their normal mean. Turns, out that sample actually had the lowest diagnostic lab activity, which isn't a surprise. It's a relative test. When you go to the diagnostic lab, they're actually using things relative to white blood cell counts. Okay. The next slide, please.

Here's some subpopulation data. I'm going to go through this really quickly but main thing I wanted to show you is the IDUAs subpopulations are pretty consistent. If you look down the right-hand column that's in red of IDUA percentages, right around 100%, all the way down, which is very unusual compared with GALC or GLA where you see some big variability.

The next slide, Laura, if you can go to the next slide, please, will exemplify this in a more visual fashion. You can see IDUA across the population on the left side of the slide, it's circled. All the results for the subpopulation are close to 100% with GALC being the worst on the right. Figures that would be the one we would have to start with.

Anyway, something of interest and I like to show is in the bottom right hand corner, you'll see a slide of GALC, which is Krabbe enzyme versus a Fabry enzyme. You can see that there is somewhat of a linear-looking response or at least a higher enzymes or equivalent for both enzymes. This is something that leads back to some of the comparative enzyme analysis. It's very helpful. The Next slide, please.

I'm going to go over this rather quickly, but you'll see first thing to notice is that the means up in the top left corner for the States where I actually had mean data are fairly consistent across the States. New York, North Carolina, Ohio and Georgia means are really similar for each enzyme. Illinois, you'll remember, has got the HPLC method and set up a little bit different, so they have different enzyme activities.

Now, look in the middle part of the table, you'll see the MPS newborns for each state. Take a look over to the far right side of that table. You'll see the IDUA all being below 5%. Just as importantly, all the other enzymes are fairly elevated, maybe one exception on North Carolina where you see a 56.1% GBA but the GAA on that is 194%. The more enzymes you're looking at, really the better at getting a picture of how serious a low-enzyme activity may be.

Then, the bottom part of the table, you see IDUA for the older patient. Look, you'll see that the IDUA activity percent of means are fairly similar whereas all the others are quite a bit lower. I wish I had a good explanation for that, but the best one I have is for potentially IDUA is more present in serum or plasma and

not just in white cells but that's just a guess off the top of my head. The Next slide, please.

Here's the summary. The next two slides summarize everything that I've pointed out in the prior slides. Roughly incident of one in 173,000, that's for the severe form. We have the non-severe case, or is to be determined but attenuated MPS I case, there's one of those.

Now, one of the interesting points is how we don't really have a lot of data to go by. It turned out that attenuated case activity was fairly low. It's hard to say, but you go to the diagnostic laboratory testing attenuated cases do have elevated residual activity as compared to the more severe forms of the Hurler form of IDUA-I.

Some other things that I pointed out. True positives have low IDUA and the other enzymes generally going to appear normal. The false positives, you'll see IDUA being low but other enzymes are generally below normal. The emphasis being I think if you want to avoid false positives running with more enzymes is really important. Okay. Next slide.

More summary. With premature infants. We see IDUA, even though in the data that I showed, you don't see IDUA being overall low in premature. In fact, it's actually slightly above normal. We do see those samples and maybe it's a sample quality issue where IDUA can be low. It seems to be a false positive because repeats come up to be more normal levels. Where it gets tricky is trying to compare that enzyme against, say, GALC or GLA. If you were to look back at the slide, I show multiple enzymes and how they're affected by premature babies. Both GALC and GLA can be two to three or four times higher than the normal mean that you'd see on the full population. You have to take that into account. I think one of the reasons why I think CLIR can be helpful is it takes all this subjective data and can make it more objective. Enzyme activities do track together. I'm pretty sure that's result of just leukocyte count variability.

We did see second tier DNA testing that would eliminate many false positives. If we were doing second tier in our lab, five of seven samples that we actually have results on would have been not referred because they were polymorphisms only.

In New York, we do plan on doing multiplex LSD testing, 6-plex enzymes for any time we have low IDUA to identify quality issues. We're thinking of using it in conjunction with border-line cutoffs. I think looking at percents and comparing to other enzymes can really help you quantify or the quality of your positive controls as well. If you've got a positive control and you look at all the enzymes, it'll help you get a better feel for how IDUA is relative to those as well.

Use caution with premature population if you're comparing with other enzymes. I think the IDUA is not so tough but as far as comparing other enzymes, it can be

tricky. Our plan is to use CLIR and keep cutoffs as a backup internal system at least initially as we gain experience with CLIR.

The last slide will show … The second to last slide. I'm sorry. If you go to the next slide, Laura. This is what I believe will be our original or algorithm once we start going live screening. I believe we'll lower our cutoffs because our 15% is obviously quite high compared with where the true positives are. Where we'll set that inevitably will depend on how things go and maybe as we see more data from other states but we will have a second tier DNA test. We do plan to use CLIR in both the analysis of our first set of samples going through as well as after we run 6-plex, which is what we're piloting now with both Pompe and Krabbe. I'll more information in the future.

The last slide is on my acknowledgements. Thank you for your attention. I did steal some information from Sarah and all the states people listed here. In the interest of time, I won't name them all but I appreciate all the information I got from them to put together summary slides. Thank you very much.

Hari Patel: Next up is Patrick Hopkins talking on digital microfluidics. Patrick Hopkins is a Newborn Screening Project Specialist at the Missouri State Public Health Lab. He was previously involved in the Missouri Newborn Screening Program for over 27 years and served as a chief from 2009 upon his retirement earlier this year. He currently serves on the APHL Newborn Screening Genetics in Public Health Committee and was a past chair of this Committee and the past chair of the APHL Newborn Screening Quality Assurance/Quality Control Subcommittee. Patrick has worked on three Clinical Laboratory Standards Institute document development committees. With that, I'll turn over the presentation to Patrick.

Patrick Hopkins: Thank you, Doctor Patel. I'm going to talk about the Digital Microfluidic Fluorometric or the DMF method, which we have used here in Missouri for over four and a half years. MPS I is included in the 4-plex screening assay that we conduct. Next slide, please.

This is our implementation plan, which was very successful so I wanted to include it here. You can see that deciding on the screening method is among the first things that you need to do and as a very important step in the implementation process but isn't it nice, though, that we have more than one choice for methods to screen for LSDs. That's not always the case in newborn screening. Next slide, please.

Just some quick facts on the DMF method. It is an FDA-cleared screening system and it has available, it's proven to be successful in a newborn screening system. There's no building modifications that were required. All we had to do was buy an minus 80 freezer to allow the rapid implementation and startup time, which can be very valuable. It's easy to use and cross train, virtually no maintenance and no instrument breakdown. The only thing really resembling maintenance is we wipe off the cartridge deck of the platform with alcohol and blow it off with canned air before we start testing each day. I've only had two platforms that

were temporarily down in four and a half years so they virtually never break down. No noise for the instruments. We can appreciate that as we get more and more noisy instruments in the newborn screening laboratory. All results are available in four hours from sample punching. This is really nice because it helps us stay caught up and enhance the timeliness of screenings. Next slide, please.

You've probably all seen this picture before of our platforms. We have two workstations. In Missouri there are eight platforms. We can easily conduct two full runs a day on all eight platforms but typically only need to do a morning run. We have plenty of room on this sidewall bench to spread out these platforms but in the next slide, this is Michigan's DMF setup. I really like how they've utilized these modular benches and have everything so compact. They're also using eight platforms. Next slide, please. This shows just another angle and how they fit four onto one workstation onto a bench along with a few other little things that they need to perform the assay. Next slide, please.

This is the 4-plex LSD kit component that includes the substrates, calibrators, controls, the extraction buffer, filler fluid, the positive controls, the drugless spot controlling cartridges. Missouri utilizes a reagent rental contract for this. Next slide, please.

The workflow for the LSD testing starts with punching, like everything else, about 15 minutes per 96 well plate. We extract the samples for 30 minutes. Then, we begin loading the samples and the reagents into the cartridge and start the test. The run time is about two hours and 45 minutes after that point. Next slide, please.

Each cartridge has its own calibration. This calibration very rarely fails but if they do, you can start another cartridge. You don't have to re-punch the 38 samples involved. You can just go back to the original sample extract and re-plate that one cartridge over again. The calibrations are the first thing the software runs in the assay. You can watch for that to come off and be assured that everything's good to go. Next slide, please.

These are quality control monitors for the run. There's two low and two medium controls are run on each cartridge. The low controls are in the effected range and the medium controls are more in the borderline or low normal ranges. Next slide, please.

These are the biochemical reactions taking place in the assay. MPS I is a substrate of 4MU labeled alpha-L-iduronidase and then the enzyme in the dried blood spot cleans off of the 4MU component, which fluoresces. Low florescence means low enzyme activity. Each enzyme reaction is able to take place in an optimum pH due to the spatial multiplexing that DMF provides. DMF keeps them compartmentalized is the word I'd like to use. This promotes optimum sensitivity for each enzyme individually. Next slide, please.

This is a typical result screen on the software showing a couple flags here. We have three cutoffs on this software. The provisional cutoff, which is yellow, is the highest cutoff, which sets us above what our actual final borderline cutoff is going to be. The high-risk cutoff for the red is the referral cutoff. Then, we have an elevated high cutoff to flag elevated outliers that need to be retested.

In Missouri, we use semi-fixed cutoffs. By this, I mean, we do have some seasonal adjustments that we make and occasional reagent lot adjustments are made. We monitor the patient mean, the number of flags we get, et cetera, like we do with all of our cutoffs in the laboratory and other enzymes. We do have age-related cutoffs because for three of these disorders, the enzymes drops as the baby ages. Ironically, that's not what we find with MPS I. That seems to stay the same so MPS I is the one that doesn't have age-related cutoffs. I'm not sure anybody knows why those don't drop. Next slide, please.

Then, after we look at the results, we do risk assessment, which includes looking at the whole picture like we do with a lot of things we screen for newborn screening. It's the baby in the NICU. We've had previous specimens on the baby, the age of the baby at collection, have they been transfused, the transit time and if the other LSDs are low. All these put together, we decide if we're going to call it high risk, which goes for diagnostic follow-up, or borderline, which requires a repeat or second screen, or inconclusive, which has a different comment because the other LSDs are low. That also requires a repeat or second screen.

This is where the CLIR tool will become helpful but as Dr. Orsini said, Dr. Rinaldo's going to be talking about this shortly. Also, a second tier test can provide additional information in this risk assessment. Next slide, please.

These are our four-year totals of screening about 308,000 births. I have included even Krabbe here, just so you can see how MPS I relates to everything, of the other LSDs that we screen for. MPS I has the second-lowest incidents next to Krabbe, applying a second tier DNA test would help sort out the pseudodeficiencies but depending on where your cutoff is, you may end up finding more carriers and variance of unknown significance as what you see with Krabbe, we find a lot of carriers with Krabbe. That one, we do have a second tier DNA test for Krabbe for the 30-kb mutation.

I guess what I'm trying to say is you don’t have to have a second tier test in place to get started. We've gone four and a half years without it but we may down the road add second tier DNA testing for MPS I and Pompe to help sort out pseudo deficiencies. Next slide, please.

I just wanted to briefly mention some striking similarities between IEF and DMF. They both have a unique and effective technologies. They have a manual sample plating component, yet can maintain high throughput. They both utilize multiple platforms for daily testing so they have built in redundancy of instruments. They both take about four hours. They both multiplex several disorders from one

sample punch. They both detect some mild or unknown variance. I know how a lot of states still use IEF, even large volume states. I just wanted to put that in there to make sure that you knew this is a high-throughput method.

Finally, I got permission from Claudia Nash in Illinois to clarify something Illinois noted in the last week's webinar in that they discontinued DMF because it was not a high throughput but they were actually trying out a 12-sample prototype at the time and couldn't wait any longer for the high throughput cartridge to come out. They made the decision to switch to mass spec at that time. Next slide.

I'd just like to thank Tracy Klug, who is our pioneer woman here in Missouri that pioneered the LSD section. She managed that section and Lacey Vermette, the senior scientist that works with her in the LSD section and all of the LSD screening team and Dr. Sharmini Rogers, Julie Raburn and Jami Kiesling and the Missouri newborn screen follow up team and our Missouri LSD task force and our contact, Tracy. My content, email, and phone numbers are up there if you need to give us a call to ask us any questions or anything like that. Thanks for your time.

Harry Patel: Okay. Next, we'll have a presentation on releasing false positives using the CLIR and second tier method by Dr. Rinaldo. Dr. Piero Rinaldo currently serves as co-director of the Biochemical Genetics Laboratory and Vice Chair of Information Management at Department of Laboratory Medicine and Pathology at Mayo Clinic in Rochester, Minnesota. He's the recipient of the 2013 Robert Guthrie Award by the International Society for Neonatal Screening and also of the 2015 March of Dimes Colonel Harland Sanders Lifetime Achievement Award in Genetics. With that, over to you, Dr. Rinaldo.

Laura: Dr. Rinaldo, please press star seven to unmute yourself. Dr. Rinaldo, please press star seven to unmute yourself. Okay. We're going to go ahead and continue with the presentation from the CDC.

Harry Patel: Okay. Thanks, Laura. The next up will be a CDC quality assurance materials by Dr. Francis Lee and Dr. Robert Vogt. Dr. Francis Lee is a member of the Newborn Screening Translation Research Initiative and Newborn Screening and Molecular Biology Branch for the CDC. He's also a professor of pediatrics at Emory University. Dr. Lee's subject matter expert for newborn screening in [inaudible 00:38:05].

Dr. Vogt is a laboratory chief in the CDC newborn screening and molecular biology branch and serves as primary investigator for the Newborn Screening Translation Research Initiative and ongoing collaborative partner with the CDC foundation. His laboratory interests focus on measuring cellular and molecular biomarkers. Since 1999, his public health interests have focused on transitional biomedical research findings and the newborn screening and other public health publications. With that, I'll turn it over to CDC.

Dr. Robert Vogt: Great. Thank you all. This is Bob Vogt. I'm here in the room with my colleagues. Francis is right next to me, Paul [00:38:51], who does our DMF assays and Chris Haynes, who has taken over the mass spec assay and Gorliz Yazdanpanah who has done any number of wonderful things for us here and has been working on the LSD for the last couple of years. Next slide, please.

A couple disclosures. I'm a federal employee and do not receive any outside funding. We have received outside funding for the last 10 years through the CDC Foundation, which is the NSTRI. At this time, we have Gorliz as the one supporter to employ from those funds. They have been donated by Biogen and Genzyme over the last 3 to 10 years. Next slide, please.

I want to run through a quick history of our support for LSD and particularly to make sure that people know who to contact now and who is no longer involved so that they won't try to reach out to the wrong person. Many of you all knew Victor De Jesus and certainly Hui Zhou. Both of them are still in our division but are not in the newborn screening branch. We miss them but they're doing well in their different laboratories. Since these changes now, the crew that is working here on support for LSD includes Paul, as I mentioned, doing our DMF assays and Gorliz and Chris, who has taken of the MS assays. Any questions regarding MS assays on a technical level certainly, you can go directly to Chris and anything regarding program level, you can track with any of us. Sophie Winchester is a young pre-doc who's an outstanding young lady. She's been instrumental in our cell culture development activities. Francis and I try to keep things in some kind of forward trajectory. Next slide, please.

I won't go through this history in detail. I will just point out that we began this a long time ago with the support from Genzyme beginning in 2005. Our first job we felt was to create the CDC reference assay. We did take the Gelb assay, which had been refined at Genzyme for higher throughput and ease of operation. Those were, for many years, the CDC reference method for looking at all the materials that we made as QC items for newborn screening. Then, we began making those reference materials. About that same time, then we began distributing the reagents for those assays, which I now called the legacy MS/MS assays. Those reagents are now exhausted except for a few archival bottles we keep around here. That program closed last year. We appreciate all the programs that participated in that and helped generate the activity that lead to the current newborn screening activities that are going on routinely now.

We did and continue to provide training and consultation to newborn screening laboratories. More recently, we've looked at developing not just analytical QC materials that would reflect all enzyme activities but condition-specific materials that could be used for proficiency testing and play began that project and did very well with it. I'll show you some data, she has presented data at APHL meetings. We'll just summarize that here particularly with respect to MPS I. Several years ago, we began incorporating the DMF platform. Chris has been working on the newer configuration mass spec assays. Most recently, we've assimilated with NSQAP and now all of the administrative things that we do go

through NSQAP. That's made things much more routinely operational. Next slide, please.

The way we make the QC pool production was originally actually suggested by Joan Keutzer at Genzyme. She and Victor worked this out. We get cord bloods from the North Carolina Cord Blood Bank, pool them. That's our 100% adjusted hematocrit, that's our QC high, our 100% cord blood. I'll mention that the cord bloods we get are excluded units and the usual basis for exclusion is low white count. Actually, our leukocyte count in our 100% QC is about the median range for newborns overall. We then use a base pool that is made from double depleted adult blood, so it contains little, if any, leukocyte content. That is the background material. Then, we admix that 50/50 and 5/95 to make our QC medium and QC low, respectively. This has been published and presented several many times. Next slide, please.

This is data from way looking at the QC material that was the lot before this one. I think we've made four batches of material over the time frame. This material was used through 2015 and 2016, I think. As you can see, in our storage conditions, in the freezer with desiccant, the enzyme activity remains stable over a four-year period. Next slide, please.

The way that we are handling QC materials now through NSQAP is different than it used to be. It used to be that you would call Victor or [00:44:34] or Gorliz and talk it out and work out a deal. Now, it's a special deal for some people. Now, it's all done through the NSQAP portal and data management team. They do a wonderful job with this. The QC materials, Francis is making things about money under the table but there is no such thing. There are two distributions each year. One in January and one in July with the other QC material. Because of the high volume and demand on the NSQAP, it's not become necessary to really orchestrate this. Joanne and her crew have done a wonderful job of that. We're assimilating completely into that program and following their lead and trying to help with it.

The default request will get you two cards of 15 DBS for each of the four levels that I've described a moment ago. You do need to request this through the NSQAP website. I think most of you all know how to do this. All requests are reviewed and subject to NSQAP approval, which is … If there a special request, then we do want to take a look at that. Importantly, the NSQAP data folks need to receive these requests two months before the ship date. That would mean around the first of November and then around the first of May, we need to know if you want QC materials for LSD and other newborn screening assays. Next slide, please.

This is the website. I think most of you all know this much better than I do. I couldn't find where you downloaded the participation form, so when I found it, I put a big red arrow there. You won't see the red arrow when you go to the website. You have to find it yourself. Next slide, please.

We have also, as I mentioned, developed condition-specific material using the EBV-transformed leukocytes from patients with specific LSD. They are made by admixing those cells into the base pool material that I described earlier. We've got a wonderful inter-laboratory evaluation of these materials. The manuscript describing that has been on my desk or in my computer for far too long. It has not yet been published. That's entirely my fault. This was [way's 00:46:58] work and it's terrific work. We want to get that into publication this year. I think that will happen. Next slide, please.

Within our laboratory using the legacy MS assays, this is what those materials look like. The normal control DBS, this is the medial activity, I believe.

Yes, I will wrap up in the next few minutes.

The Krabbe-specific and Pompe-specific and MPS-specific materials show their respective deficiencies. Next slide, please.

This further reveals the results for the IDUA enzyme. As you can see, the MPS I affected material is deficient by all four assays. The two legacy assays run on two different mass specs. The two are MS assay from Perkin Elmer and the digital microfluidics. Next slide, please.

To summarize the results that we've seen for MPS I, in the six send outs that we've conducted and NSQAP has conducted, we've looked at 162 negative results from 162 negative specimens submitted by newborn screening labs with a 2.5% false positive rate and 39 samples that were MPS I deficient by our standards. We have gotten complete agreement in all the results that have been returned to us on those materials. We think these are valid materials at least for analytic proficiency testing for enzyme deficiencies. Next slide, please.

This shows the MPS I in comparison with Pompe and Krabbe, which we have been distributing for a longer period of time. I won't go into any detail over this slide except to say that it does seem the select deficiencies are properly identified 98% of the time or greater. Next slide, please.

I want to thank, of course, the data management team. I think you all know these folks. They're in another building. You probably know them better than I do. Kizzy is actually right next door to me, so I know Kizzy really well, Kizzy and LoNeka. These folks do a wonderful job. We're very grateful to have their participation in what we're doing here. The next slide is thanks for your attention. This is Francis' selection of the baby on the slide. I just noticed that the baby exemplifies the degree of tension I expect that we should get. Thank you all very much.

Laura Russell: Okay. Now, we will hear from Dr. Rinaldo.

Piero Rinaldo: This is an outline I've tried for the sake of time. I'm going to touch on four points. I will give a very brief overview of CLIR. Then, I'll show a practical example of data processing and uploading and then, show you the specific work flow for MPS I, and then talk about the second tier test. Next slide.

Quickly, CLIR is the software that has been behind the scene for the entire Region 4 Stork or R4S. It's one we developed in 2004 for basically a quality improvement of newborn screening by tandem mass spectrometry. In the end, those things of all we decide to change basically the whole thing to the name of the software. CLIR is a multivariate pattern recognition software and an interactive web tool that allow you to really process data from multiple site. We were funded by [inaudible 00:51:14] on 2004 to 2012. Since 2012, we rely entirely on this institutional funding. CLIR has been approved by my institution as an official product of Mayo Clinic. That's why you see the three shields on that. Then, you see the URL. Next slide, please.

This is my elevator pitch about what CLIR does. It does a lot. It's a fairly ambitious but I can just read it to you. We want to replace conventional reference ranges with continuous covariate adjusted percentiles. We want to replace, indeed, we want to eliminate analyte cutoff values and instead rely on what we call conditional-specifics degree of overlap between reference and disease range. We want to enhance the clinical utility to individual markers. We do that by calculating automatically every possible permutation or ratios so if I measure two things, I have also a ratio of them.

Finally, want to replace the sequential algorithms like you have seen some examples earlier in Joe Orsini presentation. I call them the end. You do this and then you do this and then you do that. We consider the CLIR tool parallel algorithms, so when we use the concept of or. Next slide.

This is actually the reference ranges for IDUA activity adjusted both simultaneously for age and birth weight. Patrick was making some comments about the stability of IDUA. To some extent, we see that but now very early in life, you can see on the left side that the peripheral percentiles, the 99 and the first percentile showed quite a dramatic variation based on age and also birth weight. Next one. Next slide, please.

In our lab, everything from the mass spec is converted to a CSV file. In other word, any software can do it. Basically we use team review but could be specimen date or whatever used by waters. The data are converted into a comma separated value file. This is just an example of a directory we have in our servers. You go next slide.

If I open that particular file, you see I'm just showing the beginning of the end. This is file with 85 cases. We have an identifier. There are no PHIs or health information. Here, it shows the age. The age should be as granular as possible, not all 24 or 48 or 72. The birth weight, the gestational weight, these are data from Kentucky and Kentucky only provide us for less than 37, a range of one,

two weeks and anybody with a terms 37 not ideal but that's what thy can do. The gender and the six results. Here, right you see the long code. The long code is what is used to map the results of that particular analyte. Next slide.

In CLIR, we have post analytical tools. What I'm going to show you now is the tool runner. Basically, it's this is the place where you upload the file of unknown, of prospective samples. Then, you see if he gives you any hits. Next slide.

An important thing is that this can be customized at the location level and also at the individual user level. In other word, we can accommodate the system, say, somebody like Kentucky only want Krabbe, Pompe, and MPS I. That's what we provide. Others are sending us samples for all six or maybe five but not [inaudible 00:55:16]. That is very easy as long as they click the boxes and then save that as location configuration, so any user affiliated with that particular site will only run the tools they want to see. This prevents the possibility of risk of unintended findings. Next slide.

Again, if I select a file I show you earlier, it's just through click select. Then, you go to your directory. Next slide.

You can see the file has been uploaded. Then, you run the tools. Next slide.

This is high level workflow that we use for MPS I. This is a comment made earlier by Joe. I found that really a narrow number of markers is not ideal. We found that running the 6-plex actually is a good level of comfort. In other word, we get for every condition, one primary marker, the enzyme activity of [inaudible 00:56:21]. We put it through the tool runner. When we made an inquiry about the single condition, too, which is acidity for MPS I. If the score is zero, means that everything is within the first to 99 percentile but certainly above the first percentile, we call it a negative. That's done.

If any score is greater than zero and this is different than what we do for MS/MS like in RF2, where we add thresholds. Now, we say, "Anything greater than zero, we are on the dual scatter plot. The dual scatter plot is being built, we're actually compare the activity of affected patients versus false positives. False positives are here carriers and patients carrying through the efficiency alleles. If a dual scatter plot is not informative, again, case closed. If it's informative, we escalate and we repeat the analysis, adding more markers. Specifically, we add the [inaudible 00:57:24] that we measure for LD and we also, we perform a second tier test, measuring dermatan and heparan sulfate in that sample. Again, if they are normal, case closed. If we have still an informative result in an abnormal second tier test, that will be reported as screen positive. Next slide.

This is an example of a tool runner when you can see on top if you go to the next slide, there is one formative score. At that point, I go to that tab. If you go on next slide, I'm sorry. This is basically, it's similar to what I show you earlier with just some visual clarification. Basically, we are now a step two. The tool

runner tells me that there is one case as potentially formative for MPS I. Then, we go and take a look at that particular tool. Next slide.

Here is the two pages we tried to … We call it one sheet of paper so on both side, the area highlighted with the red square is actually also showing greater detail on the bottom right. The important thing here is for one thing, is everything is transparent. There are really no hidden information. It's all base shown in term of the actual values but the most important thing is the one at the bottom of the bottom left. You see in green is the covariate adjusted percentiles in red, the boxes are the actual disease range based on five known patients and the red diamond is a patient.

If you focus on the marker at the far left, IDUA, the point where I think it's important to stress is that all that empty space between the reference ranges have been green and the disease range in red is where cutoffs will fail because you can go lower and lower below the normal population but eventually, that is through the efficiency territory. Next slide.

At that point, we are on the dual scatter plot. In this particular case, we ask a question. Okay. If you have to assign this case to MPS I to a false positive, why'd you put it? Next slide.

This is actually a display of the two complete profile for two positives and for false positives. Again, you can see that they are different. Certainly, the degree of overlap for variations, for false positives is quite significant. That's exactly the pattern we want to take advantage of. Next slide.

This patient actually scores as a possible MPS I. This, for us, is the reason to escalate to very [inaudible 01:00:21]. Next slide.

This is actually, and perhaps for the sake of time, might not want to dwell too much. This is under the hood of how a dual scatter plot. It's comparing the two disease ranges, the true positive and the false positives. The area you see in yellow is where it could be either. Any value in that yellow area for each of these marker would be considered inconclusive but if a value is green, it means that it is up to level that is only seen in affected patients. On the other hand, if you see a value in the kind of pink/red area, that means is a value that is only seen in false positive. These are the information we'll combine together. Next slide.

Next step, we repeat. We do a second tier test. Next. We repeat and, again, it is informative. We'll go and take a look at this tool. Next slide.

You can see in the red box, that's what is new. It's showing you the ratios that we have calculated between IDUA and the [inaudible 01:01:39]. There's no question that this patient is above normal. As you can see on the bottom right,

it's begin to show a pattern that is not quite close to where we expect to see true positive. Next slide.

Sure enough, the dual scatter plot of a 10 plex changes the assignment. It tell us this patient is behaving like a false positive. Next slide. We're fortunate to have a second tier test for this, which I understand that people whenever talk of second tier, the talk about sequencing. I believe this is a much simpler solution. Next slide.

We have been doing it for 13 years. Quite frankly, I don't think we could live without a second tier test. According to our approach, is something we do to impose specificity. When the residual enzyme activity range overlaps considerably between true and false positive case, is of course the same specimen so there is no additional patient contact. The normal results of a second tier test overrules the primary screening. Quite frankly, it can be regionalized. It mean that you don't need every lab to do it but you can really have regions that decide to work together. Next slide.

This is not work done regionally by us. This was published by a Dutch group for a number of collaborators. We reproduce exactly this assay. Next slide.

This is what we call the scatter plot, marker versus marker, that shows where that particular patient falls. You can see it's compared to the black dots our patient with MPS I and the blue dots are false positives. We also have, you should go next slide, please, a dual scatter plot. As I told you earlier, if I had two markers, I also have a ratio. I can make a tool that basically compare the true positive on the bottom right quadrant to the false positives. Clearly, this patient belongs with the false positives.

For us, if you go to the next slide. This is just to show you again, the same comparison, you might notice here that while dermatan sulfate shows some overlap between true positive, false positive, for heparan sulfate, the middle space is white, meaning there is complete separation. There is no overlap. Next slide.

Important point here is number eight, although three out of the five things we can do were informative in the end, the second tier test overrides everything. This was reported out as normal. Final slide, please.

Our conclusion is in our hands, combining clear with a 6-plex and, as a repeat, 10-plex and the second tier test, we can achieve what I call near zero false positive. In fact, we have been testing Kentucky babies for 14 months. We had a single false positive. Was a patient with very low activity. The second tier test was normal but somewhat we decided that it was a good verification so we reported out the patient was carrier for unknown photogenic mutation. Thank you for your attention.

Laura Russell: Thank you, Dr. Rinaldo for your excellent presentation.

We'll now begin taking questions. If you have a question you'd like to ask, you can press star seven to unmute yourself or you can type your question in the chat box.

I have a question in the chat box. I believe this is for Dr. Orsini. This individual asks, "You mentioned advantages of using percent of daily mean, however, are there disadvantages to this approach as compared to percent of the daily median?" Dr. Orsini, please press star seven to unmute yourself.

Dr. Joseph Orsini: Sorry. I had the mute on on my own phone. Okay. Here we go. Yes, I believe there could be potential disadvantages if you're doing very small number of samples. The median might prove to be better than the mean although I think with IDUA the issue is a little bit less of a problem. With Pompe, you might see more of a problem or some of the other enzyme because of the variability. You get some very elevated activities in certain samples that could really throw off your mean, whereas your median would be less effective.

Laura Russell: Thank you, Dr. Orsini. Are there any questions from folks on the phone? If so, please press star seven to unmute yourself.

I do have another question in the chat box. I believe this is also for Dr. Orsini. This person asks, "In your flow chart you mentioned greater than or equal to 8 to 10% adjustment compared to other enzymes. What does that adjustment consist of?"

Joe Orsini: Yeah. I apologize that I did not have enough time to really explain that. I would be happy to show you examples if you would like to email me but essentially what we're doing is taking, I would say, is a conservative approach of using an adjustment where we see all enzymes below 100%. We exclude ASM because ASM behaves fairly funny compared with the other enzymes. Let's say, for example, the highest other enzyme is at 50%. You saw IDUA in between in that borderline range, you would just basically multiply by 100 over 50 factor to correct for that enzyme. The reason I feel that is pretty justified is those borderline ranges are typically in a range where we've never or rarely seen anybody even close to that range. It just eliminates the possibility of … Kind of an easy elimination of a false positive. Like I say, if you would like a better example, we have it in an SOP that we could share.

Laura Russell: Great. Thank you. We have another question in the chat box. "How can a state sign up to use the CLIR tools, especially if we are not screening for MPS I yet but plan to in the near future?"

Dr. Piero Rinaldo: Thank you for asking that question. Can you hear me?

Laura: Yes, we can hear you.

Dr. Piero Rinaldo: Good. Access to CLIR is contingent on contribution of data. I don't want to sound like sour grapes but I can tell you the lesson we have learned in R4S and that something I share with the secretary advisory committee recently is a lot of people use it and don't contribute anything. Now, we are asking people to make a contribution of data as a requirement to be given access. We ask for data to be submitted to us offline. We take a look, just to make sure there are no major problems. When we look at some data from Illinois, we realize we are talking about a different analyte. Joe mentioned the same thing. Their results are different from what we were seeing in other labs I've seen. I've reported this back to then. But once people have contributed data, they can rightly have access and start using the tools.

Laura Russell: Great. Thank you for that information. Does anyone on the phone have questions? Please press star seven to unmute yourself. We'll also give a few seconds for people to type questions in the chat box.

Okay. Hari, seeing or hearing no further questions, do you want to go ahead and wrap up?

Hari Patel: Sure. Thank you, Laura.

Laura Russell: Hold on. Sorry. There's one more question in the chat box. I apologize. This is for Dr. Rinaldo. When the CLIR data set changes over time, how will adjustment be monitored for reporting purposes? Dr. Rinaldo, please press star seven.

Dr. Piero Rinaldo: Okay. We monitor data submissions constantly. One of the major improvements compared to the old system of R4S is when new data are quarantine. When we are happy with the status quo, that system is not altered until we review the new data and make sure that they really fit the pattern, has been observed up to the point. There is maintenance and curation. It's behind the scenes but is going on constantly.

Laura Russell: Great. Thank you. I have another question … I believe this is for Dr. Rinaldo. "If we ever have to access old data, how could this be done? "

Dr. Piero Rinaldo: Can you hear me?

Laura Russell: Yes, I can hear you.

Dr. Piero Rinaldo: Okay. I'm not sure what you mean by old data. An important thing is that in CLIR, there are no cumulative data entries. In other words, in R4S, we ask people to calculate the percentiles and then submit the calculated percentile. Any file that you submit, you can review it and compare it to basically your own data over time or you can compare it to everybody else. It's very user friendly and very transparent about what you have in. There are several functionalities where you can compare yourself to other labs both in term of true positive and

false positives. The true positive, also, are quarantined. Nothing goes in unless it's been reviewed.

Laura Russell: Thank you. Dr. Rinaldo, we have one final question for you in the chat box. "For a lab to upload positive cases in CLIR, what data is required?"

Dr. Piero Rinaldo: It depends what you're measuring. We're asking people to basically provide everything. In fact, we're working on the comprehensive application. A complete application where people can upload everything. The immune assay, MS/MS, the lysosomal and any other things that you do, all in one single profile. Then, we can customize the location profile. Basically, our goal and it's really for the summer is to have an application where you don't have to jump from MS/MS to lysosomal to congenital hypothyroidism is all together. The user defines what they want to see. Once they upload that file, it will run top tools, they are customized from that particular location. If you run lysosomal and you do 4-plex like New York, you do 5-plex, like Tennessee or 6-plex like us. That pretty much what we say what you should upload, all four, all five, all six, whatever you have but without leaving anything out that you actually measure.

Laura: Great. Thank you. At this time, we're going to go ahead and wrap up. I will turn it over to Mr. Hari Patel.

Hari Patel: Thanks, Laura. Just want to let everybody know that this webinar, along with the previous two webinars will be archived on the APHL Newborn Screening training web page. Participants will receive a link to this page in the post-webinar follow up email. P.A.C.E. credits will be awarded for attending this webinar. To receive P.A.C.E. credits, participants must complete the post-survey evaluation, which will appear in the post-webinar popup window and follow up email. If anyone has any questions, they can contact Laura Russell and their contact information is displayed. I'd just like to thank everybody for their attendance for today's webinar. Thank you.