DIFFERENTIAL DIAGNOSIS AND TESTING FOR HEMATOLOGIC MALIGNANCIES

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MODERATOR Welcome back from lunch everyone. Hope it was tasty and you got a little sunshine. We're going to go ahead and get started, but before we do, if you could all take a second to make sure your phones are silenced. Our first lecture is on the differential diagnosis and treatment for hematologic malignancies. It's my great pleasure to welcome our fabulous speakers Dr. Sandy Kurtin and Dr. Jennifer Knight from the University of South Carolina, School of Medicine. Thank you.

DR. KURTIN Good afternoon. I know it's really tempting to be out there wiggling your toes in the sand. So, thank you for coming inside and joining us this afternoon. It's been a really, really, really good day for heme, don't you think? Great talks and so those of us are committed to these hematological malignancies or maybe do them a little bit, I think it's been a really great meeting, so far. So, we're hoping, you know – we heard a lot this morning and even yesterday, about the importance of diagnostic results, in terms of both predictive and prognostic indications. And so we're hoping we can back that up a little bit and look at this process of differential diagnosis and testing and why it is so critical to do this in the best way possible. Our goal really is to talk about some of these newer techniques for diagnostics, and now the mandate you saw in the

session on myeloid malignancies the – you know, how much these criteria – the ELN criteria were edited just for AML. And if you've looked at the World Health Organization diagnostic criteria, the most recent update, which is 2016, from 2008, it reads a little bit like a chemistry textbook. It's gotten very, very complex. So, hopefully, we can touch on – we're not going to really go into so much that level of detail, but really how do you answer those questions? So, these are our disclosures.

The first thing is really talking about this concept of precision hematology. How do we keep up with this robust science? We know that most of these diseases, you saw again earlier today, that slide with the heterogeneity of the myeloid malignancies, and that makes a big difference in what we see in terms of clinical presentation, and also variability in survival. We heard, in the drug updates, just a short while ago, about, you know, even looking at mycosis fungoides in Sezary cell and how you were trying to differentiate drug toxicity with disease itself. So, variable presentation, variable survival, based on these prognostic indices and the ever-changing characterization of these diseases. So, really staying up with this has become critical in what we do with these patients.

I think, at least, in pathways and targets. Right? And we know that there are these key pathways. Some of them have actionable targets. And, even if they don't yet have actionable targets, believe me, there are a lot of people working really hard to try to make them actionable because that's how we change the natural history of the disease. So, thinking about these key pathways, what do those pathways do? What do they do when they’re abhorrent or mutated? And,

then how do we exploit those targets for therapeutic benefit? So, remember when this was this simple. Right? Like the hematopoietic tree and you have lymphoid and you have myeloid and they grow up and they become red blood cells, white blood cells and platelets, or T or B lymphocytes. And we all thought it was just that easy and wouldn't that be swell? Right? Well, now it's this. And this is even just a light rendition of what we're thinking at. So, now we're beginning to think of, what are the growth factors, and that's something we've looked at before and we've been able to administer some of those growth factors to manipulate that particular cell line. But we also know that there are now transcriptional factors that are involved and it's not as simple as just the cytokine that's involved in that differentiation and basically maturation of that cell. So, we are looking at transcriptional factors within each of those cell lines and each of the processes of maturity of that individual cell. And so again, thinking in that way.

And then we take it a step further and this is one of the beautiful illustrations from our supplement on CLL and our initiative of Priming the Pump, something that I came up with, to really begin to prepare us, to prime our pump. We are the pump. We're priming that pump to really begin to think in pathways and targets and so as all these new agents are coming out, we're like, "Okay, yeah. That's an IDH1 inhibitor. That's an IDH2 inhibitor. That's what that pathway does. Here's another drug in that class." And we can begin to incorporate this into what we do every day, because it's not going to get any easier. So, we have the opportunity to optimize our diagnostic process now for these potential therapeutic targets, but we need to ask the right questions.

So plan ahead. Those of you who may have participated in the bone marrow biopsy workshop – that was one of our big – the first step one, what's the question? What sample do you need to answer what you think your question might be, so that our hematopathology colleagues have the ability to basically answer that question for us. So, how many samples do you need? What tube does it need to be? If we need an excisional biopsy, so we are going to get tissue, more tissue the better. More to look at. So when we're looking at nodal biopsies, you know, we had a colleague, Sean Hehn and Dr. Tom Miller, who I worked with for many, many years, who wrote a paper, many years ago now, about FNA versus excisional biopsy and lymphomas. And understanding that the architecture of a lymph node is very complex, and literally a needle in a haystack when you're doing FNAs and you see a pathology report come back that says, lymphoma. Like, "Okay, tell me a little more about that." Because there's 33 different types of lymphoma and in order to make that diagnosis, you need a good amount of tissue. So, really understanding what's the best way to answer that question when you need a surgical biopsy and then certainly, with the bone marrow biopsy and aspirate, what the characteristics of that are.

So, let me just use one example here. This is polycythemia vera, one of the myeloproliferative neoplasms. And, just to have you familiarize yourself a little with this World Health Organization classification system. So, this is an example of what those criteria look like for this specific disorder. There are major criteria and there are minor criteria and we know, obviously, we can get, you know, hemoglobin and hematocrit off of peripheral blood, but we do need to know

cellularity in a bone marrow. And then we need molecular mutations. We're going to talk a little bit more about how you can get there using different techniques. But in this case, JAK2 V617F is the most common abnormality in polycythemia vera and then less commonly, roughly 3%, have the exon 12 mutation. So understanding those criteria are very important.

We then are going to look at what are the characteristics within these MPNs of these different mutations? What other questions might we add to that? So, if we know that JAK2 is 95% of their cases, but there are those few that don't have that, what are the other questions that need to be answered. So, familiarizing yourself with this criteria, what are the other abnormalities that might be present in these diseases like MPL and CALR? Very important. And rarely we have these patients – I have a patient right now who's triple negative. Doesn't express any of these but has clearly myelofibrosis based on just the morphological appearance of the core biopsy in the marrow. So, familiarizing yourself with the criteria, that's just one example of what you would be looking at.

So, in terms of a bone marrow biopsy and aspirate, first of all, we're going to look at maturation. Are there blasts? Are the cells in the right numbers and along – do you have trilineage hematopoiesis, meaning all of the different cell lines? Are R routes present? That's diagnostic for AML, generally. What's the morphology? This is where it really takes a skilled eye on the part of the hematopathologist, to really detect dyspoiesis, meaning abnormal shape or size. And this is very critical to a diagnosis such as MDS.

We're going to look at flowcytometry, we'll talk more about that. Cytogenetics. PCR, polymerase chain reaction. Gene expression profiling. And in anybody who's had an allogenic stem cell transplant, we need to get chimerisms.

Our clot section – so that basically is our first pull. And when we do a bone marrow, we're going to look for spicules, those are the little cells that tell us, basically, that we're in the marrow space. The excess of that first pull goes for a clot section, which can really provide us a lot of information, so again, understanding what do you need from that sample? Then we do a core. And the core is also very important. This is decalcified in the lab. But, before they send it off, they get little tweezers and they do little touch preps. Sometimes they do it with a slide. But that tells us other information about the architecture of the marrow, what's the cellularity and roughly, your cellularity is 100 minus your age. So, that's easy to remember. I'm not liking what 100 minus my age is getting to be. All of a sudden, it's like wait a minute. I was doing a thing earlier and 65 was old and I'm like, "I'm sorry, that is not old." So, but 100 minus your age. Let's not get off track. So, the architecture tells us a number of things, myeloid versus erythroid. You may see M:E ratio on that report that you see. You're going to look for, you know, presence of immature precursors. The marrow is normally very logically organized and things can be off where they shouldn't be, so there's something called atypical localization of immature precursors, or ALIP, that can be seen in certain instances. The presence of fibrosis, the only way you can really see that is on the core. And then, if we're looking for other solid tumors, you know, that would be another thing that we could learn from this. If you have a

dry tap and you can't get an aspirate, and then you take two cores, because we can tell a lot from that, not everything.

So, let's look a little bit at the other tests and then Dr. Knight is going to go through our Tumor Board, if you will. So, I love this slide. This is from a previous paper, some time ago, and I call this the tapestry of B lymphocyte disorders, and you can see the difference in each of these little squares that a hematopathologist needs to really apply an algorithm, basically looking at preparing those slides, various stains, to get a sense of morphology. Are the cells big, are they large? Are they white blood cells? Are they red blood cells? Are they platelets? But, it really takes a trained eye to be able to detect what this primary diagnosis might be. So, really, it can be done – part of it can be done fairly quickly, but it's not unusual to call and say “What do we know?” And they say, "You know what, it's not really clear. We need to do some additional stains." So, in that algorithm, this can actually take a little bit more time.

Then, there's flow cytometry and this is something that's really evolved, where you heard earlier about the new multi-parameter flow cytometry, which has become much more precise, but Dr. Miller, who I worked with for many, many years, talked about this as the zip code, and these CDs, not the things you play on a CD player, if we even have those anymore, but these are clusters of differentiation and they basically are these cellular proteins that help us, you know, where you can say a certain malignancy expresses CD10, CD5, etc. And sometimes these CDs are targets for therapy. We heard about brentuximab CD30 and – it's not always necessarily diagnostic by itself, but it helps us paint

the picture of what this disease might be. Thankfully, it can be done both on blood and on tissue, so we can send peripheral blood for flow sometimes in CLL, where there are a lot of cells circulating. We can get a lot of information from that. We can send bone marrow. We can send tissue, but it needs to be fresh. So, processing that tissue becomes very important.

And then we have metaphase cytogenetics, which, you know, is the typical, you know, your 46, XY, 20, you know. We look at 20 metaphases. That's what's in the little bracket when you look at a report. The cells must be actively dividing, because they're metaphase cytogenetics and so there is some times that we miss things here, and cells that aren't dividing. This is something that still requires bone marrow, most of the time. There are a few exceptions, in AML, when they have like a bazillion blasts in the peripheral blood. We can sometimes detect it in that way. But generally, it needs bone marrow.

The other thing it's going to tell us is the numeric details, so ploidy. Is it hyperdiploid, too many? Hypodiploid, too few? Trisomies. Are there deletions, duplications, translocations, inversions? And that gives us all that information. And sometimes, as you can see here, this is the World Health Organization criteria for AML with myelodysplastic-related changes. If you can't make the diagnosis based on the other details of morphology, or other testing, there are certain cytogenetic abnormalities, which by definition, imply that that diagnosis is present. So, really, again, being able to, you know, ask the right question, run the right tests. If we want to get a little more precise, we can go to FISH, fluorescence in situ hybridization, and basically this is another way to identify

chromosomal abnormalities that may be missed by metaphase cytogenetics. One of the best examples here, is in a disease-like myeloma, where you have fully matured plasma cells that aren't necessarily dividing. Right? They are already grown up to be what they're going to be. And you may get normal cytogenetics, but by FISH, you can actually detect some of those very important cytogenetic abnormalities, like 13Q or 17P, and such. So, it can help us understand that. It can help us also look for gene mapping and also ploidy determination.

The bad part, so it can be effective, we can do it on various tissues, we can use this to follow people over time, so that's very useful. The drawback is, you need to know what you're fishing for. So, when we say, go fishing, FISH for, you need to know you're going to order FISH for myeloma, or FISH for CLL. You don't just order FISH, because they are very specified panels and you need to know what you're looking for.

Then we have PCR, which is the next level. And this is just basically amplifying that further. So, if you just think about your, you know, your microscope is getting bigger and bigger and bigger, not truly, but in thinking that way, and so this is amplifying further so we can detect the structural abnormalities as well as the single base pair abnormalities. This is highly sensitive. It's fast. It's really great for things like testing BCR-ABL over time when we're trying to figure out what's going on with a CML patient, for instance. But, again, you need to know what you're looking for. You have to be able to ask the

question. It's just not a general panel. It can be done, also, on fresh tissue or formalin-fixed paraffin-embedded tissue, so FFPE.

And then the last piece of this is next-gen sequencing, and you hear a lot about it and everybody thinks they want next-gen sequencing and it's this big new fancy test. It's very expensive up front to develop these little libraries that basically allow you to test for a panel of these genes. And, until very recently, not all of these test – well, still today, not all of these tests are covered and there are definitely some potential drawbacks there. These are very recent publications, looking at approval for next-gen sequencing for ALL and in pediatric patients and multiple myeloma, so that the FDA has recognized these as tests that can be covered, for minimal residual disease measurement or measurable residual disease, as we heard in the earlier presentation.

So, with that, I'll turn it over to Dr. Knight to lead you through the Tumor Board.

DR. KNIGHT So, good afternoon. As Sandy said, my name is Jenny Knight. I'm a hematopathologist from Greenville, South Carolina, and we thought it would be useful to work through a couple of real-life heme-malignancy cases with a focus on the genetic testing.

So, we can jump into our patient. This was a 65-year-old man who had been referred to oncology for a PET-positive pancreatic mass, as well as multiple lytic bone lesions. He came with the presumed diagnosis of metastatic pancreatic carcinoma. Some labs that were available the day of his appointment showed that he was significantly anemic. Hemoglobin of 6.2, as well as thrombocytopenic

with platelet count of 100. His metabolic panel that was available showed he had renal insufficiency and he had a significant protein gap of greater than 8. So, in this patient with lytic bony lesions, anemia, renal insufficiency, and a large protein gap, it's understandable that the oncologist was concerned about myeloma as well as this pancreatic mass. So, we wanted a biopsy of both: one of the bone lesions, as well as the pancreatic mass. And, what's shown here is a picture of one of those lytic bone lesions and it shows small cells infiltrating, actually, out into the soft tissue around bundles of skeletal muscle. And this is different than what I would expect to see in a metastatic pancreatic carcinoma. I would probably see some malignant gland formation. Here, I just see individual, single cells. And this was morphologically by H&E, very suspicious for plasma cells. And that was confirmed with immunohistochemical stains. You can see the small picture down in the corner is a CD138 immunohistochemical stain that confirmed these as plasma cells, and so the lytic bone lesion actually turned out to be plasmacytoma, and luckily biopsy of the pancreatic lesion just turned out to be chronic pancreatitis.

So, in this patient where anemia, renal insufficiency, imaging showing multiple lytic lesions of the bone, this biopsy-proven plasmacytoma, now gets us to a diagnosis of symptomatic myeloma, even without a bone marrow biopsy.

So, the next steps for workup of this patient, with myeloma, would include genetic testing. And you can see in the box here I have highlighted, that not only does NCCN stay metaphase cytogenetics or a karyotype, but also a FISH panel. So, why both? It's what Sandy was talking about earlier. Plasma cells do not

grow well in culture and so that karyotype will often be negative, even when you detect abnormalities by FISH. So, don't be surprised if there's a discordance there. But the reason for continuing to do the karyotype as well, is sometimes you may detect large abnormalities in the chromosomes that were not specifically targeted the FISH. Remember, FISH studies, you're only going to find out an answer for the mutations that you're specifically looking for.

One more thing, I just want to point out on this slide, is the mutations that you're looking for in the FISH panel, not only is it translocations, which is marked there by the little T, but also deletions and amplifications are important in the setting of myeloma, and I'll come back to that in just a second.

So in our patient, we have the tissue that gave us the diagnosis, that plasmacytoma biopsy, so are we going to be able to get the genetic studies that we want with the tissue that we already have? And what we have is that formalin-fixed paraffin-embedded tissue. When it's embedded in the paraffin like that, it's a state that we can store the tissue for many years. It's also at a state from which that we make the slides. And so since the tissue has been fixed in formalin, karyotype is not possible. That requires fresh, viable tissue to grow in culture. So what about the FISH panel? Well, some FISH is able to be performed on FFPE tissue. Some FISH testing is not able to be performed on FFPE tissue and this goes back to what I said in the last slide about, not only are we looking for translocations in the setting of myeloma, but also deletions and amplifications. And the significance of that has to do with the fact that the way FISH is performed on FFPE tissue. It starts with cutting a very thin slice of the tissue,

about 4 to 6 microns thick, or 4 to 6 one-thousandths of a millimeter, and then placing that tissue slice on a slide to which the fluorescent labels are then added. So, if we think about that top picture as our 3D cell embedded in the paraffin wax, and that yellow cylinder is the nucleus and those two green dots are one gene. Remember, we have two alleles for every gene. So, if the section that we take for our FISH slide cuts through where that red line is, when we place it on the slide, it looks like there's a deletion because we only happen to hit one alleles of the gene going through. So, that's why when you're looking for deletions and amplifications, or copy number variability by FISH, FFPE tissue is not a good option. So, in this case, if we want genetic studies on our patient, we are going to have to get additional material, so the source that would make the most sense would be a bone marrow biopsy. And they did. They performed a bone marrow biopsy in this patient, but of course, it turned out to be a dry tap. So, now what do you do?

So, the tissue that we get from the bone marrow biopsy is across the top, core biopsy, test preparations, aspirates and clot sections. So since it was a dry tap, we don't have the aspirate. And that's what's usually sent for genetic testing. We don't have the clot section but that's formalin-fixed paraffin-embedded tissue so we wouldn't have been able to use that anyway.

What we do have is the core biopsy and the touch preparations, both of which can be used to help us get the genetic studies that we need. So as Sandy mentioned, you can get a second core. You would send the first in formalin for morphology, just as usual. But if you take the second core, and keep it fresh,

make sure you don't put it in fixative. You can put it in RPMI or some other tissue culture media. Or, if you don't have that available, putting it in some normal saline would be just fine. And then what happens when we get it in the lab, is that we will just aggregate it, or literally shake the cells off of the bone, like shaking the leaves from a tree. And the cells that fall off into solution can then be used just like the aspirates, or the genetic studies or even flow cytometry, whatever is needed.

The other thing you guys could do is additional touch preparations. Those additional touch preparation slides can be used for FISH studies and the touch preparation slides are different from those FFPE slides, in the fact that when you touch the core, on the slide, the whole cell is transferred onto the slide. So, rather than just that thin slice that we got with the FFPE tissue, we have the whole cell and so deletions and amplifications can be looked for just like translocations.

And finally a third option, which is usually not an option in myeloma, but if there happened to be circulating plasma cells, then you could potentially send a peripheral blood sample, as well.

Patient two, this is an 81-year-old woman who had presented to her primary care physician with some shortness of breath, weakness, and fatigue after she had returned from a trip to Italy. She was also complaining of some hip and back pain. Her past medical history was significant for osteoporosis and atrial fibrillation, for which she was taking aspirin and clopidogrel. Her primary care physician sent her for CT angiogram just to exclude the possibility of a pulmonary embolism after she had developed the shortness of breath from her

long trip. And so, that was negative for PE, but did end up showing significant lymphadenopathy, as well as some liver lesions and also an x-ray of the hip showed a pathologic fracture. So, this patient's imaging studies were highly suspicious for lymphoma. So, the patient was sent to the orthopedist first, in order to have the hip repaired. And, the hope was that a lymph node biopsy could be performed during the hip arthroplasty surgery in order to get a diagnosis. But, unfortunately, there was no lymph nodes in the operative field, and so only the femoral head was obtained. So in ortho surgery, not surprisingly was reluctant to go in and do an open biopsy, if they didn't have to in this patient, and suggested interventional radiology. But unfortunately interventional radiology did not want to perform a biopsy because the patient was being anticoagulated. So, the poor oncologist calls Friday afternoon to see if there's any way that a diagnosis could be made available over the weekend so that she could start treatment on this patient. So, we thought about what material did we have available to us, which was mainly the femoral head from the surgery that had been performed that morning, but slides would not be available from that until Monday. Because it was a bony specimen, it was going to have to be decalcified in order to process it appropriately. So what we thought is maybe we could dig out some of the marrow from the medullary area and desegregate it for flow cytometry. We also considered the possibility of flow cytometry of the peripheral blood. If they were circulating lymphoma cells, maybe we would catch those.

So here's flow cytometry from the femoral head and this is the box that should be showing B cells, which are CD19 positive, but unfortunately no cells,

no B cells, were detected and it was nondiagnostic. So, we neither ruled in nor ruled out lymphoma with this test. The peripheral blood flow cytometry did find a B cell population. It's highlighted there in that first square, of a CD19 positive. Those little blue dots are all the B cells. And when looking at the light chains, you can see that they are all expressing the same light chain. They are all kappa positive, so this is a monoclonal B-cell population, that further immunophenotyping showed it to be CD5 positive so it was consistent with a CLL-like immunophenotype. It only accounted for 1.8% of total cells, though. And so, in this patient, this was very suspicious, but it's also potentially a red herring if it's just a monoclonal B-cell lymphocytosis in a woman who has something else going on. So, we had to wait for the slides to come out on Monday to get a more definitive diagnosis. And here, I'm showing the cut surface of the femoral head itself, on the left, as well as a low-power view of the histologic section on the right. And, you can see, in the medullary bone, there is a lot of variation in color, grossly and even on the slide. And if we zoom in to look at those variable areas, we see on the left, at the bottom, that pink stuff is necrosis and above it, adjacent to that, there are some areas of diffuse, large atypical cells. Other areas showed some normal hematopoietic elements, but those were sprinkled with these small lymphoid aggregates. Both abnormal and both needed to be further evaluated with immunohistochemical stains. And, so that's what I'm showing here. The slides across the top are representing the large cell areas, and the slides along the bottom are representing those small lymphoid aggregates. And so with immunohistochemical stains, both populations proved to be CD20-positive B

cells. The small lymphoid aggregates also expressed CD5, so they were similar to that monoclonal B-cell population identified in the peripheral blood, CLL/SLL. The large cell population was actually CD5 negative. But, in the end, what we diagnosed was chronic lymphocytic leukemia/small lymphocytic lymphoma, as well as diffuse large B-cell lymphoma and, in this lady, likely representing just transformation.

But to further talk about diffuse large B-cell lymphoma and high-grade B-cell lymphomas, in general, a little bit, is this is a group of diseases that has a lot of variabilities still and behavior in response to treatment, such that subtypes are being parsed out so you can better follow these patients. This diagram here shows that if we look across the top, not only does morphology play into the final diagnosis of our high-grade B-cell lymphomas, but also immunophenotype and cytogenetics. So, morphologically, hers looked like diffuse large B-cell lymphoma, but one option for a subtype of that is the high-grade B-cell lymphoma with MYC and BCL2 and/or BCL6 translocations, or in other words, the double or triple hit lymphomas, which are defined by a translocation of MYC, a translocation of BCL2, and/or a translocation of BCL6.

All of these are actually available by immunohistochemical stains, but overexpression by immunohistochemistry has been shown in multiple studies to not correlate very well with the genetic findings and so those studies really are needed for a diagnosis of double or triple hit lymphoma.

So, in our patient, if we decided that we needed further subclassification of this large B-cell lymphoma, would we be able to get the answers that we needed

from the diagnostic material that we already have? So, what do we have? We have the femoral head. We have the formalin-fixed paraffin-embedded femoral head, and this time all we're looking for is translocations. And so double and triple hit lymphoma panels are validated in FFPE. Unfortunately, this was a bone. It had be decalcified. Decalcification is an acid-based process that denatures DNA and so renders it useless for any genetic testing. So, that is not an option in this patient. The peripheral blood was positive too, but only for that low-grade CLL component. So, we would not be able to look at that for the double or triple hit lymphoma genes if that's what we were interested in. So, if we did want to do further genetic testing in this woman, we would have to get another sample, and options would include a bone marrow biopsy to either send the aspirate or, this time, clot section could be sent. Potentially, a lymph node biopsy and if it had been known at time of surgery, the hip surgery, that there was going to be such difficulty in getting diagnostic material on this patient, it's possible that you could see if the orthopedist could do an aspirate while he's in there, or even potentially arrange for a bone marrow biopsy at the time of surgery for staging purposes in this presumed lymphoma patient.

So, our final case today is going to be a 68-year-old man who was initially admitted to the GI service for a significant anemia. Hemoglobin of less than 6. And the urgent care center sent him over to the hospital with a presumed diagnosis of an acute GI bleed. So, the GI team knew he had recently had a colonoscopy and so that's a lower GI source, was unlikely, and they performed an EGD in the hospital and that was normal and so an upper GI source for an

acute bleed was also excluded. But, if we look further at his CBC, it's not surprising that a GI bleed was the source of his anemia. His hemoglobin was low, but so was his white count and his platelets. And, he had a significant neutropenia and circulating blasts. So, this is actually highly suspicious for acute leukemia. So, a bone marrow biopsy was performed, and not only did it show blasts but it also showed cells along the morphologic maturation spectrum toward monocytes. And you can see that left picture is blasts and where those are seen in the peripheral blood. The center picture are promonocytes, or they're a little bit more mature than our blasts, but are considered blast equivalents. They have a somewhat folded or convoluted nucleus. And then on the right is mature monocytes. And so once the immunophenotype was confirmed with flow cytometry, the patient was diagnosed with acute myeloid leukemia with monocytic differentiation.

Here's the flow cytometry. And just as there was a morphologic maturation spectrum toward monocyte, I can see that immunophenotypically, as well. The cells that we saw, that I just showed you on a previous slide are represented here by all the blue and green dots and they have that spectrum from mature – or from immature to mature, where the green dots are mature monocytes and the one on the right shows CD34-positive blasts. There's white and blue cells that move up into CD34-negative monocytes.

But for final subclassification, I can't just say this is acute monoblastic leukemia or acute monocytic leukemia, NOS. First, I have to do genetic testing to determine final subclassification. And so, NCCN guidelines recommend here,

genetic testing. They had cytogenetic analysis listed as karyotype +/- FISH. Here, notice that they dictate both karyotype and FISH, and the difference with this and the plasma cell myeloma is that unlike plasma cells, which don't grow well in culture, blasts do grow well in culture. And so, if you get a good karyotype, with a full 20 cells, then typically FISH is of no added benefit to the karyotype. Molecular studies, however, are important and are becoming more and more important. The NCCN has those 8 mutations listed there that they recommend testing for in all acute leukemia patients. But if we look at the footnote, the highlighted area, it says, "This field is evolving rapidly." And I can't stress that enough. "This field is evolving rapidly." And so they acknowledge that there are other platforms out there to look for different mutations other than the ones that they have listed, including next-generation sequencing panels. And those panels may detect mutations that affect prognosis for your patient. Those panels may detect mutations that affect therapeutic decisions for your patient. Those may detect mutations that affect whether or not your patient can go on clinical trial. So, the question becomes, should all of your AML patients be tested with next-generation sequencing panels?

So, this is the part of the presentation where I will frustratingly leave you with lots of questions and no answers. Okay? This is something I think that just needs to be considered on an individual basis with your treatment team, with your patient. What genes in these mutation panels are significant for prognosis, diagnosis, therapeutics? Can these mutations in the panel that I'm considering be used to follow my patient over time? Other questions that may not be at the

forefront of your mind, that need to be considered, too, is what is the turn-around time for these studies? What specimen do I need to send in order to get the result that I want? And, just to keep in the back of your mind, will the insurance company pay for the testing that I want?

So, this is the slide just showing some of the panels that I found, looking on the Internet, from some of the National Reference Laboratories. I have no affiliation with any of these labs, but it does give you just a sample of what's out there to choose from. The one from NeoGenomics covers 28 genes up to the one from Foundation Medicine, which looks at over 400 genes. Similar to all of them, though, is turn-around time. Most of these next-generation sequencing panels are going to have a turn-around time of close to 2 weeks—something to keep in mind when you're making decisions about whether or not to order these.

The other thing to look at on this slide is for the acceptable specimens. Yes, all of them will accept peripheral blood or bone marrow aspirate specimens, but you can see a couple of them listed up there, will also accept formalin-fixed paraffin-embedded tissue. So, the significance of that is that these don't necessarily have to be performed up front, that they can be performed at a later date if you change your mind and decide that you do want some of the information from these panels.

So, just to look at a little more closely those questions I posed in the previous slide. What is diagnostically significant? So, some mutations dictate subclassification of AML. APL with a 15;17 is kind of a prototypic one, but there are molecular mutations that define subtypes of AML too. AML with NPM1, AML

with CEBPA. There are some mutations that are less diagnostic specificity though. As such, for example, the ones that are associated with clonal hematopoiesis of indeterminate potential.

When considering prognosis, this has to be considered in conjunction with the karyotype. Much of the literature that is out there is based on prognostic significance of the mutations in the setting of a normal karyotype. So, how will an abnormal karyotype affect the prognostic results of the mutations that you're looking at. Should you consider the NGS testing after you get your karyotype back? With it being able to be performed on FFPE tissue, that's a possibility.

And also, what about the different combinations of mutations within the panels? Again, a lot of the information available is done on one specific mutation, the prognostic significance of one specific mutation. So, if there are more than one mutation found in your panel, how do those things play together? Are they additive? Are they synergistic? Do they cancel each other out? Just things to be considered.

And, finally, what about the significance of allele frequency? So, what about the proportion of mutated leukemic cells, is that prognostically significant? Does it matter if one or both alleles in the gene are mutated? So, for like CEBPA for example, biallelic mutation is what is associated with good outcomes versus FLT. It's been more recently shown that the mutated to wild-type ratio of greater than 0.5, is what is associated with worse prognosis. For many of the genes, information is still being gathered on does allele frequency have significance?

When it comes to therapeutics, you guys know better than I do that there are more and more drugs coming out every day that are available to target specific mutations. Will the turn-around time of 2 weeks for some of these next-generation sequencing panels affect your ability to act and use some of those medications? If it's something you're considering for induction therapy, then NGS panel might not be the way to go.

Also with regard to enrolling your patient in clinical trials, but also whether or not they will respond to the treatment and whether or not the insurance company is going to pay for the treatment, is a consideration with allele frequency.

And finally, a little bit about MRD in the setting of the next-generation sequencing panels, but also in general. MRD in AML is a different beast than it is with ALL or myeloma. When we think about these genetic mutations and being able to follow them over time, obviously if the patient doesn't have a detectable mutation at diagnosis, then following that every time becomes a moot point. But, if they do have a mutation that's detected at diagnosis, we know that some mutations can be followed for minimal residual disease. We know that some mutations can't be used to follow for MRD. For many, however, the answer has not been found yet, and that's being worked on. There was a recent article from the *New England Journal of Medicine* that proposed a different strategy of rather than looking at individual mutations over time, to look at the next-generation sequence panel as a whole and at later dates, if there is any mutation found, that

would be considered MRD positive rather than following each individual mutation over time. So, more and more information being collected in that regard.

Allele frequency becomes significant here in the fact that many of the commercially available next-generation sequencing panels, at this point in time, have an estimated limit of detection, up to 5 to 10%. So, that's really not that low if you think about it. And usually at a level that would be detectable either morphologically and/or flowcytometrically.

When is the right time to test? Okay. That's well-defined for some things such as APL, when you know that you can't look for the 15;17 translocation until after consolidation for it to be significant. This was highlighted in an article I found that showed, in their study, a third of the patients that had detectable disease at day 14, later had no detectable disease, even without additional therapies. So, what is the meaning of that detected mutation, at that time point? And, frustratingly, there is always going to be patients that have MRD, that don't relapse, or patients that don't have detectable residual disease that do relapse.

If detectable disease is found and you are able to change your treatment based on that fact, is that going to change outcome? I think that's the ultimate goal and ultimate question that is still left to be answered for many of these studies and these treatments.

And just one last thought, as a pathologist. This is a relatively new testing modality, testing platform, and so with all the labs developing their own different panels, just be aware that there is lab variance.

So, to follow up with our patient, he actually did have a next-generation sequencing panel sent. And, you can see the mutations that were tested for are across the top there. And no mutations were detected. They did note, in the report, that their established detection limit of allele frequency was 5 to 10%. In this patient, he actually had some other results that came back first. He had FLT3 mutation analysis that was sent individually. The treatment team was hoping to start midostaurin as early as possible and so could not wait for the 2-week turn-around time of the FLT3 and NGS panel. And that actually came back as low-level positive. Below the limit of detection of what could be seen on the NGS panel. And his karyotype actually came back with a poor prognostic finding with the monosomy 7. So, in just seeing the patient to keep, to look at the whole available information when making treatment and management decisions for him.

So, I just would like to leave you with these thoughts and when you're deciding about what genetic testing to order is, will it affect diagnosis? How will it affect prognosis? Will it be able to change the way I treat or follow this patient? And then what specimen, obviously, do I need to send in order to get the information for my patient? This field is rapidly evolving and so I encourage you guys to keep an open conversation with pathology. We love hearing from you. That way we can focus our efforts to answer the questions that you have rather than putting information out there that you guys don't really find useful.

And with that, I'll say thank you for your time.

DR. KURTIN So, very compelling thoughts. I think also within the context of the other hematological presentations we've had, but certainly also in

the context of cost. So, I think really fine-tuning what we do and making sure that the questions that we are asking get us what we need, but aren't necessarily just so we have every bit of information that may not be actionable.

So, we have some time for questions. If there are any questions. Let's see. Anybody have a question? There's one over there.

QUESTION 1 Hello. I just have a general question about blood testing for, you know, like instead of doing tissue biopsies for any variety of cancers, are there any, I know they're starting to do a lot of stuff with just blood. Is there any one that's like better or are they validated? Are people really using that?

DR. KNIGHT So, I think that's an area that is in its infancy. A lot of times, you have the same questions with that that you do with some of these other tests. If you find a mutation, does that necessarily mean the patient has disease and, you know, there's MGUS, there's monoclonal B lymphocytosis, there's clonal hematopoiesis of indeterminant potential. So just because you find an abnormality doesn't always necessarily mean you have a specific diagnosis. So, I think there's great potential there, but it also needs to be kept in context.

QUESTION 1 Okay. Thank you.

DR. KURTIN Any other questions?

QUESTION 2 Hi. I have a question about the FLT3 analysis, because I know I've sent out, as well, just the single mutant, to University of Michigan -- gotten a positive. But then our next-gen sequencing got me a negative. And we discovered with those two assays, is that the next-gen

sequencing panel, they base pair – mutant that you can detect. It's only up to 67 base pairs. Whereas if you send it to the University of Michigan, they can detect up to 400 base pairs. So, if you have a mutation that's larger than the average, you're going to miss it on your next-gen sequencing.

DR. KNIGHT Mm-hmm. I think that's very important and what comes in to play with when I mention the lab variance and stuff. These panels and the primers that they choose to use for the panels are going to vary from lab to lab and from test to test, and so you may run into that. And so, just need to find out through trial and error, kind of what is best to get the answers that you need. And so, maybe individual testing, for at least that one specific gene, for now is the way to go.

DR. KURTIN The other thing that's really intriguing is I was just recently at a meeting, I don't know, about a month ago with all the world experts in AML, in particularly in FLT3. And, you know, when you're looking for FLT3 ITD, that may be detected by next-gen sequencing but there are other types of FLT3 that are not. And actually FISH ends up being a better test for that, so I think this is another example where the science has really rushed ahead of our ability for clinical utilization and so to your point of the nuances of next-gen sequencing, that science is still fairly early and those libraries that are being created, you know, basically using these probes, are variable across the different products. So, it's an evolving science, as you saw on one of the slides, there.

QUESTION 2 Thank you.

DR. KURTIN Yes.

QUESTION 3 So, 7 years ago, I scared the hell out of cancer and it took both of my breasts. I had no symptoms, no signs to suggest breast cancer. I had ductal carcinoma in situ in my right breast, lobular carcinoma in situ in my right breast, stage 0. In my left breast, I had ductal carcinoma in situ, stage 0 and stage 1A. My Oncotype DX score was a 7. I didn't have to do chemo or radiation. I had to take letrozole for 5 years. So, now, I feel like my purpose in life is to advocate and educate other women and men that are going through breast cancer. I had to take this mic this afternoon to tell all of you all in here, as a person who has experienced breast cancer, as a mother, a military wife, and a survivor, it means more to me than you all will ever know, the fact that you're sitting here, in this room, doing what you do, not just today, but on a daily basis. So, don't think that there are patients out there that don't appreciate you all. Thank y'all from the bottom of my heart. And if you want to read my story, go to boldandbreastless.com. Y'all have a blessed day.

MODERATOR I think with that, we'll say have a blessed day. Thank you.

[END]