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Guest: Dr. Melissa Snyder is co-director of the Antibody Immunology Laboratory at the Mayo Clinic in Rochester, Minnesota with an academic appointment as Associate Professor of Laboratory Medicine and Pathology.

Randye Kaye: Hello, and welcome to this edition of JALM Talk from the *Journal of Applied Laboratory Medicine*, a publication of the American Association for Clinical Chemistry. I'm your host, Randye Kaye.

Vasculitis is a group of autoimmune disorders characterized by inflammation of the blood vessel walls. Some types of small vessel vasculitis are associated with the presence of antineutrophil cytoplasmic antibodies or ANCAs in the blood. ANCA detection in the clinical laboratory by indirect immunofluorescence or IFA represents an important tool in the diagnosis of these disorders so that patients can be identified for appropriate treatment. This IFA test may be performed on ethanol-fixed neutrophils and antibodies staining patterns are detected visually. However, the presence of other antibodies in the blood, specifically anti-nuclear antibodies or ANAs, can lead to an apparent perinuclear ANCA or P-ANCA pattern on IFA, potentially causing false positive result interpretations.

A focused report in the January 2022 JALM Special Issue on autoimmune diagnostics describes a study that investigated the association of various ANA patterns and concentrations with P-ANCA interference. The data provided from this study may aid laboratories and result interpretation and interference mitigation for ANCA testing.

On today's podcast, we are joined by the corresponding author of this article, Dr. Melissa Snyder. Dr. Snyder is co-director of the Antibody Immunology Laboratory at the Mayo Clinic in Rochester, Minnesota with an academic appointment as Associate Professor of Laboratory Medicine and Pathology. Welcome, Dr. Snyder.

Melissa Snyder: Thank you for having me today.

Randye Kaye: Can you start by telling us more about what is ANCA and what is its clinical significance?

Melissa Snyder: Sure. So, ANCA stands for antineutrophil cytoplasmic antibody. This means that these are antibodies that recognize antigens that are found in neutrophils. The ANCA testing is performed using immunofluorescence testing and this is done using neutrophils that have been fixed in some way. Usually ethanol fixation is the most common method.

There are two primary clinical applications for ANCA testing. One is in the context of vasculitis and these would be the small vessel vasculitides like granulomatosis with polyangiitis, all those kinds of diseases. In the context of vasculitis, we know that the antigen that is recognized by the ANCA, at least in some of the ANCAs, is myeloperoxidase. And when a patient has an antibody against myeloperoxidase, which again is found in the granules of neutrophils, that produces a perinuclear or P-ANCA staining pattern on the immunofluorescence on the neutrophils. The other clinical application for ANCA testing is with inflammatory bowel disease, and this is a little bit different than we see it in vasculitis in the sense that patients with inflammatory bowel disease or some patients with inflammatory bowel disease, will have an antibody that looks like a P-ANCA pattern on the fixed neutrophils. The difference is though in that the patients who have inflammatory bowel disease, this P-ANCA is not generally associated with a myeloperoxidase antigen specificity. And in fact, we really don't know what the antigen specificity is in patients who have inflammatory bowel disease. But we're still looking for this ANCA perinuclear pattern on the fixed neutrophils in order to assess for this antibody.

Randye Kaye: So, I think you've already started to answer this next question but, what else can you tell me about what prompted you to perform this study? Like what questions were you aiming to address?

Melissa Snyder: Sure. So, we know in the laboratory that we will often identify this perinuclear or P-ANCA pattern and we don't find a corresponding antigen specificity such as the myeloperoxidase that I mentioned before.

So, the question really then is, what causes this? Why do we see this P-ANCA pattern and not find an MPO antibody? Well, one reason is that the antibody might be against another antigen that's present in the neutrophils and that there a lot of different antigens in the neutrophils that could be causing this type of pattern. We don't test for all of those individual

antigen specificities in the laboratory but it could certainly be a reason why we would detect this P-ANCA pattern without seeing a myeloperoxidase antibody. But another reason that we can see this is the presence of an interfering antibody and specifically the interfering antibody we mostly are concerned about is the presence of an anti-nuclear antibody or ANA.

Now, probably many folks listening to this are familiar with ANAs. They are used in the context of connective tissue diseases, often as a screening test for patients where they have a suspicion of lupus or Sjogren's syndrome, something along those lines. But what's interesting about ANAs is that they can sometimes look like a P-ANCA pattern on those fixed neutrophils. And so, the question then becomes: when you see a P-ANCA pattern on these fixed neutrophils and you don't know for sure that they have a myeloperoxidase antigen specificity, what do we do with this P-ANCA? How do we report it, especially if we suspect that it could be associated with the presence of an ANA? A laboratory might still report it as a positive P-ANCA; they might report it as a negative because they believe that it's actually an anti-nuclear antibody instead of a true P-ANCA; or they might report it as indeterminate.

So, we wanted to look at ANA interference on P-ANCA testing as a way to, maybe learn something about this interference, and maybe think of ways that we might be able to mitigate it in the laboratory, as well as providing the most accurate information for the patient medical record.

Randye Kaye: Okay. So, very important study then. So, can you summarize some of your key findings?

Melissa Snyder: Sure. So, let me just talk a little bit first about how we actually set up the study. So, what we did was to select the cohort of samples, close to 400 samples, where we had testing that was performed for ANA testing by immunofluorescence or by the IFA, and we selected a cohort of negative and positive samples, and among the positive samples, we included a range of titers as well as a variety of different patterns. We then took all of those samples that we had collected and tested them by another ANA method which is the enzyme immunoassay. We also tested all of these samples on our fixed neutrophils to look for whether or not we would identify a P-ANCA pattern, and we also tested them for the myeloperoxidase antibody specifically because we were presuming that, again, most of these should be myeloperoxidase antibody

negative, and that if we see a P-ANCA pattern, we would attribute it to the presence of an ANA.

So, what did we find? So, if we look at the samples that were negative on the ANA testing, regardless of whether it was negative on the enzyme immunoassay or negative on the immunofluorescence assay, we found about 5% of those samples showed a reactivity for the P-ANCA pattern on the fixed neutrophils. Now, this wasn't surprising because we do know that the P-ANCA pattern can arise from antibodies other than an ANA. So, we would presume that these samples have an antibody against something in the neutrophil, it's just not one of the ANAs.

We then looked at the ANAs that were positive on the enzyme immunoassay and we found that there was an increased frequency of P-ANCA interpretation on our fixed neutrophils and that was in the range of about 20% to 25%. So, almost a quarter of the samples that were positive on our ANA enzyme immunoassay showed reactivity or showed an interference, essentially, on our ANCA testing on our fixed neutrophils. But what was a little bit surprising is that this wasn't correlated at all with the strength of the reactivity on the ANA testing. In other words, the stronger ANAs, the higher semi quantitative results didn't show more reactivity on the ANCA. So, it was just kind of it was positive by the ANA on enzyme immunoassay, we saw about a 25% positive on our ANCA testing.

The same was not the case on the ANA testing that we did by the immunofluorescence. So, we found that -- and if you look at samples that were positive by the immunofluorescence ANA test, about 13% of those samples showed an interference on the ANCA immunofluorescence testing. However, this was correlated with the titer of the ANA by immunofluorescence, and what I mean by that is samples that had a higher ANA titer showed a higher rate of interference on the ANCA testing. That was the first finding. The second finding is that on the ANA by immunofluorescence, we also report a pattern. And when we stratify the samples based on pattern, we found that certain patterns were more likely to cause an interference on the ANCA testing specifically the homogeneous pattern showed almost a little over 30% interference rate on the ANCA testing, whereas the dense fine speckle and centromere all were less than 10% interference on the ANCA testing.

In addition to that, the very high titer homogeneous samples showed a very high interference rate: almost 85% percent of the samples that were high titer homogeneous pattern demonstrated an ANCA interference on those fixed neutrophils. So, it tells us that ANAs are an interference on ANCA testing, and that it is related to the specific ANA that you're looking at; and we make that interpretation based on the pattern that we're seeing by the ANA on immunofluorescence.

Randye Kaye: Wow. So, how do you suggest that other laboratories can incorporate those findings into their practice?

Melissa Snyder: You know, I think it kind of depends on whether the laboratory does the ANCA testing in their lab or whether they have it as a send out. For those laboratories that perform ANCA testing, I think that this is useful information to help guide them as they troubleshoot P-ANCA patterns in their laboratory. They may choose to run an ANA by IFA on samples where they observe a P-ANCA pattern but don't detect the myeloperoxidase antibody. They might choose to do an ANA by enzyme immunoassay depending on what's available in their laboratory. But it does provide some evidence to suggest that when you have a P-ANCA pattern that you can't explain with the antigen specificity of the myeloperoxidase, then I think doing an ANA test to help understand the nature of that immunofluorescence that you're seeing on the neutrophils could be useful.

If your lab doesn't do ANCA testing, if you have it as a send out, I think this is still important information for you to know, you really need to be understanding how you best interpret a P-ANCA result when you don't have a myeloperoxidase antibody result or you have a negative finding on the myeloperoxidase antibody. And I think it also is important to understand when you sometimes will see reports from a laboratory that says P-ANCA indeterminant, then it's good to understand why that laboratory might be reporting it as indeterminate, and it could be an indication that they suspect that the patient's reactivity is actually due to an ANA rather than a true P-ANCA that might indicate that some additional testing would be warranted to help really understand the autoantibody profile for a particular patient.

Randye Kaye: All right. Thank you so much. Very interesting. Thank you for joining us today, Doctor.

Melissa Snyder: Thank you. And I would just like to also acknowledge the co-authors on the study. Nick Larkey was a fellow in the Mayo Clinical Chemistry Fellowship when he did this project. He's now an Assistant Professor at the University of Virginia. And Ashley Denome who was a Clinical Laboratory Technologist in our laboratory at the time. She's now been promoted to a Technical Specialist. I want to acknowledge the efforts that they put it on this study.

Randye Kaye: That was Dr. Melissa Snyder from the Mayo Clinic, discussing the JALM focused report entitled "Correlation of ANA Characteristics with P-ANCA IFA Interference." This article is part of JALM's January 2022 Special Issue entitled "Autoimmune Diagnostics, Fundamentals to Cutting Edge." Thanks for tuning in to this episode of JALM Talk. See you next time, and don't forget to submit something for us to talk about.