

August 22, 2022- Patient Education Series: The Diagnostics of Pemphigus and Pemphigoid

Becky: Welcome, everyone. I'm Becky Strong and I am the Outreach Director here at the IPPF and I will be your host for today's webinar. Thank you for joining us. Today, we are joined by Dr. Raminder Grover, Laboratory Director for Beutner Laboratories for today's Patient Education Webinar. This call is now being recorded.

I would like to thank you for being on the call with us and to our Sponsors Sanofi Regeneron, Genentech, and argenx, and for making today's call possible. "Information is a key factor in treating and living with any condition. However, every patient's situation is unique. The IPPF reminds you that any information found on the internet or during presentations should be discussed with your own doctor or healthcare team to determine if it applies to your specific situation." Let me introduce you to our speaker for today.

Dr. Raminder Grover is the Laboratory Director for Beutner Laboratories, which is run and operated by KSL Diagnostics. She oversees all laboratory operations including diagnostic testing and quality management systems, consults with physicians, and provides leadership for the delivery of clinically useful laboratory services. With extensive experience as a microbiologist and a virologist in India before moving to the U.S., Dr. Grover received her certification from the American Board of Medical Microbiology (ABMM). Joining Dr. Ernst Beutner to train in skin immunopathology, she soon developed an immense interest in autoimmune bullous skin and mucous membrane diseases. She specializes in the diagnosis of autoimmune bullous and connective tissue diseases by direct immunofluorescence tests and serology studies. Dr. Grover spent nearly 10 years training and working with Dr. Beutner, who pioneered and established the diagnostic tests for this group of rare, disabling diseases. She is a volunteer faculty member in the Department of Dermatology at SUNY, Buffalo, and has authored research papers in skin autoimmunity, microbiology, and virology.

Before we begin I would like to go over a few housekeeping items. For those of you who are on their computer for this webinar, you will see on your screen that you can access the audio either by using your telephone to call in or from your speakers and mic on your computer. As you see on my screen right now you have the option to select one or the other. Please be sure to select the method that you will be using. If you would like to ask a question please type your question into the text box under the questions section in your GoToWebinar sidebar. We received many pre submitted questions prior to the webinar as this is a very popular topic. We will try our best to answer as many questions as we can within the hour. On the webinar today we will be discussing the diagnostics of Pemphigus and Pemphigoid. If you ask a question that does not pertain to the webinar subject I will have to ask you to email me after the webinar. For those of you on the call that aren't on the web, you will not be able to ask a question. So if you would like to ask a question, please click on the link that was provided to you in your confirmation email.

Now, it is my pleasure to introduce Dr. Grover to discuss the diagnostics of pemphigus and pemphigoid and answer your questions.

Dr. Grover: Thank you Becky for your kind introduction and for the invite to discuss a diagnosis of Pemphigus and Pemphigoid and thank everyone for being here today. Becky, are we able to see my screen?

Becky: Yes, you should be able to, to see your slides.

Dr. Grover: So the diagnosis of these, rare but potentially, lethal and disabling diseases has come a long way. since Dr. Beutner and Dr. Jordan first described that autoimmune blistering disease could actually like Pemphigus Vulgaris could be caused by our own antibodies. Dr. Beutner and his colleagues standardized, established and implemented the immuno fluorescence tests that we still use. And the first evidence that a blistering disease was caused by autoantibodies was provided by Dr. Beutner and Dr. Jordan, in 1964 right here at the University of Buffalo. They demonstrated the binding of IGG to the B N Z to the Pemphigus and Pemphigoid patients to the epidermal cell surface and a few years later the binding of antibodies was seen to the BMZ in Bullous Pemphigoid patients. So their first report demonstration of skin antibodies in sera of Pemphigus Vulgaris patients by indirect NATO fluorescent staining has a time, 885 citations. Both Dr. Beutner and Dr. Jordan were honored with dermatology foundations discovery in the year 2000 for their contributions and immuno dermatology. And many of their discoveries actually led to the development of new diagnostic tests that we still use for blistering diseases. And we will be discussing some of these tests today.

So, before we go into the diagnosis of these diseases, let's talk briefly about the pathogenesis of both Pemphigus and Pemphigoid. Both these diseases are autoimmune and are caused when your immune cells or B cells start making antibodies that bind to the body's own proteins or antigens. And these antigens are localized in the epidermis are the dermal epithelial junction in case of Pemphigoid and the epidermis in Pemphigus. Now the skin is composed of three layers.

We have the epidermis, the dermis and the hypodermis. The epidermis consists of cells known as keratinocytes, which are bound together with desmogleins and desmocollin. And these are two important proteins to hold the cells together, And in Pemphigus, our body starts making antibodies to desmogleins. And in Pemphigoid the antibodies are targeted to various proteins in the dermal epidermal junction. You'll see the details of the dermal epidermal junctions here in this diagram. There are multiple proteins that help in this binding but some of them are involved in a bullous pemphigoid group of diseases. Such as BP180 and BP230 are involved in Bullous Pemphigoid. Antibodies to laminin 332 can be seen in what was initially known as epilydin autoimmunity. Now we call it laminin 332 Pemphigoid. Antibodies type seven collagen are seen in a condition called epidermolysis bullosa acquisita or Epidermolysis bullosa and antibodies to laminin Gamma one are seen in laminin gamma one pemphigoid and we will be talking a little bit about all of these conditions in our slides later. So what happens in Pemphigus? We see the desmosomes here, they are finding the cells together. The antibodies to desmoglein 1 and 3, come sit on these desmogleins. And they are no longer able to hold the cells together. The cells fall apart and you start seeing intraepidermal blisters or blisters within the epidermis. In Pemphigoid, the antibodies target the junctional area. They come and sit on structures called hemidesmosomes or other proteins in this area. And this

triggers a strong immune response. And all these immune cells, then start secreting chemicals or enzymes which destroy these four things, And the epidermis splits up from the dermis is the glue that's finding them together. It is no longer there. And it just gets stripped off. Giving our a sub epidermal blister which is typical of the Pemphigus group of diseases. So the diagnosis of these conditions is basically multi-step. And it involves three major tests, histology and direct immunofluorescence, and serology. Histology and immunofluorescence are performed on a biopsy.

In fact, histology probably, would be the first test that the clinician would request or do when he suspects it to be an autoimmune blistering disease. And then direct immunofluorescence is done to demonstrate the binding of the tissue bound antibodies. And then, depending on the results of histology and direct immunofluorescence, serology of blood work is done to look for circulating antibodies and also to find out the target auto-antigen. So we now know that the autoimmune blistering diseases are categorized into two major types: intra-epidermal or the Pemphigus and other subepidermal where we see blisters within the epidermis and sub epidermal blistering diseases where we see a split below the epidermis or the Pemphigoid group. Histology, DIF, and serology is indicated for the diagnostic work up, for both the group of these diseases. And the characteristic pattern of immune deposits seen in direct immunofluorescence, guides us to do the serum tests like what serum tests are needed after we do the DIF studies. For example, when DIF is indicative of Pemphigus, the serology would include, Indirect immunofluorescence tests on monkey esophagus, followed by ELISA Studies or desmoglein one, and desmoglein three antibodies. If clinicians suspected it to be paraneoplastic pemphigus, besides doing indirect immunofluorescence on monkey esophagus, and desmoglein one and three ELISA,. IIF on rat bladder is indicated and also specialized tests for an endoplakin and periplakin antibodies that are needed. So if the DIF indicates that it is a Pemphigoid group of disease, then the serology would be done on Monkey esophagus salt splits skin, followed by ELIZA, depending on what we see on salts split skin. I'll be talking about these later.

Now going onto histology. So light microscopy biopsy studies can give us the first information on the differentiation between Pemphigus and Pemphigoid. The observation of intra-epidermal microvesicles or sub-epidermal separation and the presence or absence of inflammatory cells helps us to make the differentiation. But the DIF and serology are indicated for a definitive diagnosis. So far, histology. The dermatologist or the dentist that you go to would collect about four mm punch biopsy from the edge of the blister. It's transported in 10% formalin and submitted to the laboratory for testing. And the sections are then examined under a light microscope. It's important to collect the edge of the blister because it gives us a better visualization of where the split is. And so we see here in Pemphigus, histology can also help us to differentiate between the two major forms of Pemphigus. The characteristic histo-pathological finding in Pemphigus Vulgaris is a suprabasal blister split. So, we see here that the superficial layers are intact, whereas the basal layer has split up and sometimes we can see a single layer of cells lining the space. And these cells are known as tombstoning

cells. The upper epidermis is intact, whereas the opposite happens in Pemphigus Foliaceus where the superficial layers are split up and the bottom layers or the basal layers remain intact. So the reason for this is explained in the next slide here.

So, and as we know, that both Pemphigus Vulgaris and Foliaceus are caused by a lot of very simple issues are caused by antibodies to desmoglein 1 and 3 or combination or just one of them. So these coatings have a variable distribution in mucosa and in the skin. So, in Pemphigus Foliaceus which is predominantly a DSG-1 antibody mediated disease, the blisters I've seen in the superficial skin and also not in mucosa because desmoglein three, which is present in high quantities in the upper, in the deeper epidermis. And throughout the mucosa compensates for the loss of desmoglein one and in Mucosal Pemphigus Vulgaris antibodies against desmoglein 3 are predominant. So this causes blisters only in the deep mucosa and not in the skin because we have desmoglein 1 here, which compensates for the absence of desmoglein 3.

In the mucocutaneous form, we have antibodies to both desmoglein 1 and desmoglein 3. So we see lesions in both skin and mucosa.

So talking about the histology of Pemphigoid. In Pemphigoid the early lesions can show epidermal edema with inflammatory cells and some eosinophils. But there is nowhere clear-cut visible split between the epidermis and the dermis. In advance of these regions we can see a sub epidermal blister, and this blister contains serum, fibrin, inflammatory cells, which are predominantly eosinophils.

So now going on to the most important part for these diseases, direct immunofluorescence. Now, for rolling out autoimmune blistering diseases. As we've seen besides clinical and histopathological examination, it is very important to demonstrate the presence of tissue bound immunoglobulins or complementary components. And the tissue bound immunoglobulins or immune deposits can be detected by direct immunofluorescence. So, for this test, skin or mucosal biopsies are taken and it is frozen in sections of cut and let's say in this diagram, let us assume that this is our skin biopsy with antigens in it. And we have the autoantibodies which have gone and bound to the skin antigens. Now, this binding of autoantibody with skin antigens, like desmogleins or BP 180 or 230 and is detected by adding or incubating skin section with the primary antibody which is linked to a fluorescent epifluorophore known as FITC. So, if the auto antibodies are present in the skin, this primary antibody will get bounded here and we will see fluorescence under the epifluorescence microscope. If the patient's

biopsy does not have this autoantibody, this primary antibody will not bind and will get washed away in the large step and we will see no fluorescence. So this test is done for IGG, IGA, IGM complement and fibrin deposits in our lab. And these are all different types of isotypes of immunoglobulin. And Complement C three is an important immune component, and we test for fibrin deposits also just to see how regional, or if the biopsy is appropriate for ruling out the autoimmune diseases for sure.

The sensitivity of a direct fluorescence is greatly influenced by the biopsy site. So the ideal site for the biopsy specimen for DIF studies depends upon the autoimmune disease that needs to be ruled out. Sometimes multiple biopsies may be needed. It is important not to take a biopsy from the base of the lesion from the bullae or erosion. The immune deposits can be degraded in these areas, and therefore it will give us a false negative result. So at least a 3 to 4 mm punch is needed to detect the in-vivo-immune deposits in relevant areas, because sometimes the deposits may be focal. And we should never cut the biopsy into two for both DIF and H & E studies. Separate biopsies should be taken because the pressure, due to cutting, can lead to artifactual split or it will really, split the epidermis away from the dermis. And that's not helpful for ruling out bullous pemphigoid or pemphigus. And for transport, biopsy specimens can be snap frozen in liquid nitrogen, the best is to transport it in a special medium known as the Michel's medium. Normal saline can be used for up to 24 hours. Sometimes we've observed that biopsies connected to normal saline give less background, but we have to get it to the lab within 24 hours. And immuno-reactions have been detected in Michel's medium for up to six months. But we advise testing within 10 days because it gives us the best results and do not use formalin for direct immunofluorescence. In our retrospective study them, at our lab, we found that biopsies positive for pemphigus vulgaris begin to lose immunoreactions within two minutes of immersion in formalin and the biopsies for pemphigoid begin to lose their immune reactance within 10 minutes. So, whenever a biopsy for the DIF's needed, it's important that we have Michel's medium sitting right there so that we do not put it in formalin. So that we generally like to send this inflammation to physicians, when they are collecting biopsies and this chart here is the diagram here, describing the biopsy sites, ideal biopsy sites for the conditions we are trying to rule out. So to rule out Pemphigus and Pemphigoid and EPA lamina in 382, autoimmunity and Gestational Pemphigoid or Linear bullous disease, to skin biopsies are generally recommended for the DIF, one from the edge of the lesion, and 1 about 3 centimeters away. But if your physician chooses to do only one biopsy, then this would be the best where we have a part of the lesion, and a part normal site. We do not want to go here, because for the reasons I described before, that inflammation can really lead to a negative DIF finding. And two biopsies suddenly needed from the lesions. One 3 to 10 mm away from the lesion, and one, from a regional area to rule out erosive like lichen planus because, most of the times, these conditions can mimic each other. So we need to test for both at the same time. So a normal biopsy, a normal area and a regional area should be biopsied. And we're not talking about DH dermatitis here. But again, we need a biopsy from a normal area for this condition.

DIF is still considered the gold standard, and its most widely used test for rolling out autoimmune blistering diseases. And it can give us an accurate diagnosis if the following parameters are used for interpretation. So the lab should generally report this primary site of immune deposits. Is it present in the epidermis or the dermal epithelial junction?

What is immunoglobulin? ISO Type C, IgG, IgG4, or subclass IgA, IgM complement or fibrin? What is a patent or deposits? Are they linear granular or are they fibulure as we see it Dermatitis Herpetiformis? Are there multiple immune deposits present? And if so, what is the identity of the most intense deposits? And sometimes the pollution can be seen in sites other than the main site. For example, in PNP, Pemphigus Erythematosus and DH, and I will show you some pictures of these later. In Pemphigus Vulgaris, DIF gives us the characteristic epidermal cell surface or the intercellular in all forms of practices. This is due to the binding of autoantibodies to the desmosomal proteins in the interstellar space. Most cases reveal IGG; sometimes focal CT deposits can be seen in the epidermis. The deposits are generally continuous but sometimes granular deposits can be seen and in our experience, IGG four subclass gives stronger deposits than IGG in a lot of cases. So we recommend testing for this subclass as well. And sometimes, I know there are studies which report that there can be variation in intensities of immunofluorescence seen in Pemphigus Vulgaris and Pemphigus Foliaceus. But in our experience, the deposits are transepidermal. And we generally cannot distinguish between the two forms on DIF. We rely on the serology for DSG1 and DSG3 antibodies to differentiate between these two forms and also on histology because that can determine the level of the split. And in certain, some situations, a mix of both IC and BMD deposits can be seen. And the first scenario is Pemphigus Erythematosus where immunopathologicaly UVC of Pemphigus Foliaceus and lupus erythematosus. We can see intracellular deposits, as well as some deposits in the dermal epidermal junction. Although a lot of these patients do not have lupus. They will just show this finding on DIF. And another condition is Paraneoplastic Pemphigus. They're both intracellular and the basement membrane zone deposits can be seen because these patients have antibodies to multiple autoantigens. And also when we test for IgG subclasses IgG1 subclass shows up stronger than IgG4 compared to Pemphigus Vulgaris and pemphigus foliaceus where we see more of IgG4 and weaker IgG1.

Pemphigus Vegetans is another condition where we see histology very different from Pemphigus Vulgaris. Most of these patients show antibodies to desmoglein 3 but also, along with those desmoglein3 antibodies to desmocollin 1, 2 and another protein called periplakin are present. And if instead of IgGA intercellular or cell surface deposits by direct immunofluorescence, it indicates IgA Pemphigus. And IgA Pemphigus can be of two types: Superficial, where deposits as seen in the superficial epidermis or transepidermal. Their deposits are seen throughout the epidermis. And most of these cases are negative for desmoglein 1 and 3 IgG antibodies. Patients with subcorneal pustular dermatosis are superficial type of Iga Pemphigus and can have antibodies to the desmocolling 1, 2 or 3. And

patients with IEN type or the transepidermal type of IGA Pemphigus have antibodies to multiple auto antigens including desmoglein 1 and 3

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But these are IGA types so they will not be detected by the regular ELISA's. And most patients are positive with DIF. Whereas circulating antibodies are seen only in about 30 to 65% of patients.

So, what is a sensitivity of direct immunofluorescence? Direct immunofluorescence shows a sensitivity of 90% to 100%. False negatives can be seen if the biopsy is from an inflamed area or the base of the blister, or there are some technical problems in the lab that tested it. Sometimes the tissue may not be adequate and we may not have enough epidermis to examine. Or even if it's placed in formalin before it's transferred into Michel's medium, it will read negative. Can also be negative in drug induced Pemphigus and also in certain PNP cases, either in very early lesions where the lichenoid changes are predominant or if we are given an ...area or a perilesional area, deposits will not show up here.

25:45

The specificity is 100% because false negatives are very rare in the DIF, one should be aware of some non-specific staining that can be seen in the lab sometimes but then IgG4 helps to rule that out. In our experience when we see here is the default itself of IgG, then rerun IgG4 is completely negative. So, that helps us to rule out any false positives that we may see. And in these situations where direct immunofluorescence is negative, but, you know, the clinician strongly suspects that it is Pemphigus. Repeat DIF should be advised and also serology is indicated to further rule out these conditions. So these photos here show us examples of intracellular deposits in the epidermis. We can see this patient fishnet-like pattern here or chicken wire pattern and the first figure is the photograph of IgG deposits and you can see that G4 is so strong here it shows up much better than what we see here in Figure C. Figure C has a focally granular pattern. Something that we could easily call negative if this IgG4 test wasn't run. So another example here is showing a weak IgG weak focal which can easily be missed but becomes, you know, the test becomes much better if you've done IgG4. Here we can see intracellular deposits in the epithelium.

And this is a legional area where we see that the epithelium is separated from the lamina propria with some free cells here. And there's hardly anything that we can see in the epithelium as far as inter cellular deposits are concerned.

So going into IgA Pemphigus. So basically we are dependent on histology and DIF to rule this condition. As of now, we don't have any serology available commercially for this form of Pemphigus. So in our lab, DIF is reported within 24 to 72 hours and our report includes the type of antibody we see and also the localization of the antibodies observed. So the report would typically say the causes of IgG and IgG4 were observed in cell surfaces or intracellular areas of the epidermis and the result would be, the findings are consistent with Pemphigus. Sometimes, if we see the positive, I like reporting it as suggested but not diagnostic because it may be an early lesions that has been biopsied or the biopsies from an earlier legion. Then we recommend doing a repeat biopsy. And if a repeat biopsy cannot be done, definitely serology is indicated and that may give us some clues that yes, we have circulating antibodies and then we can go back and repeat the DIF if needed. The positive predictive value of DIF is 100% because as I mentioned before, there are no false positives here but the negative predictive value is not 100% because we can see false negatives of this test in Pemphigus.

So, going on to the serology of Pemphigus. Serological essays helps us to demonstrate the circulating antibodies, and also they provide us useful information regarding the auto reactive antigen. So, two commonly used tests for Serology in Pemphigus are indirect immunofluorescence and ELISAs. In indirect immunofluorescence this principle of fluorescence is the same as indirect immunofluorescence in biopsy studies. But here, instead of the biopsy, we are using a substrate which has antigens like desmoglein or desmoglein depending on what antibody we are trying to detect. So, we have the substrate here and this triangle here represents the antigen sitting on the substrate. And this inverted Y is an antibody in the patient's serum. So this is the autoantibody that we want to detect. So we react the patient's serum with the substrate and say for example if we are trying to detect IgG antibodies, we will take anti-IgG antibodies which are represented here in orange, also known as secondary antibodies. And this is conjugated with FITC. Now if the patient's serum has autoantibodies they will bind to antigen sitting in the substrate on the slide. And when we wash this after this step, it will stay here. And when we add secondary antibodies, these antibodies will bind to the patient's antibody which is sitting on the substrate here on the slide. And when we examine under the microscope, we will see fluorescence. If there are no antibodies in the patient's serum, there will be nothing for this orange antibody to bind on the slide and there is no fluorescence seen. So now the use of substrate depends on what condition we are trying to rule out, because and most commonly used substrates are monkey esophagus, guinea pig, esophagus, and rat bladder epithelium. From the labs we do not test or antibodies on Guinea pig esophagus anymore. We just run monkey esophagus along with desmoglein1 and 3 ELISAs

The reason being because we compared the sensitivity of differentiating between Pemphigus Vulgaris and Pemphigus, using both these substrates along with the ELISA, we found that there wasn't much difference in running two substrates versus running just monkey esophagus. Especially after that ELISA's of desmoglein 1 and 3 became commercially available and FDA approved for our Pemphigus Vulgaris and Pemphigus Foliaceous. Now the sensitivity of IIF on rat bladder epithelium, definitely useful for ruling out PNP. So basically, it depends what your clinical is suspecting, and the use of substrate will depend on that. And in both the labs we test for IgG and the IgG 4 in routine and IgA and circulating autoantibodies are also tested. If you're trying to rule out IG of Pemphigus, but the sensitivity of this test is not that high. And as I mentioned before, ELISA's are commercially available for ruling out some, but not all autoimmune blistering diseases.

So the Serological diagnosis of Pemphigus Vulgaris and Pemphigus Foliaceous. The indirect immunofluorescence on monkey esophagus or Guinea Pig esophagus and normal human skin has reported tends to yield 70% to 95%. Generally titers of the intercellular antibodies are reported, and endpoint titers are given in the report for IgG and IgA4 antibodies. And Elisa for Dsg1 and Dsg3 antibodies have a very high sensitivity tends to be specificity ranging from 96% to 97%. These ELISAs help to confirm the diagnosis and also how to differentiate between Pemphigus Vulgaris and Pemphigus Foliaceous. So in Pemphigus Foliaceous we see antibodies to desmoglein 1 in Pemphigus Vulgaris antibodies to desmoglein 3 are seen and in Muco- cutaneous form both Dsg1 and Dsg3 antibodies are seen. So the results are given in ELISA units, and ELISA for Dsg 1 and 3 is more useful for monitoring disease activity compared to IIF. However, the titers or ELISA units may not always correlate with disease activity, and high index values have been seen in patients in remission.

So this is the micrograph of IIF done on monkey esophagus epithelium. And we can see the honeycomb or chicken net-like pattern of deposits with IgG in patients of Pemphigus.

So I would like to talk a little bit about Paraneoplastic Pemphigus because we need to do special tests to rule out this condition. And Paraneoplastic Pemphigus is an autoimmune disorder, linked to an underlying lymphoproliferative disorder. It was first described by Dr. Anhalt in 1990 as an autoimmune entity distinct from Pemphigus Vulgaris. Patients exhibited distinct autoantibodies against desmoplakin 1 and Bullous Pemphigoid 230-kd antigen. So this condition has a strong association with the hematologic malignancies, which account for about 84% of these cases. Although other malignancies have also been noted in PNP.

So in PNP, besides, desmoglein 1 and 3 antibodies, which we see in Pemphigus vulgaris and in Pemphigus Foliaceous, patients can have antibodies to Desmoplakin 1 and 2, Endoplakin, Periplakin, BP230 and plectin. And this is known as a Paraneoplastic Pemphigus complex. However, about 16% of patients may not demonstrate antibodies to proteins in this complex.

So the defining features of PNP are very painful oral lesions and polymorphous cutaneous eruptions. Lesions can cause blistering, lichenoid changes or like erythema multiforme type changes. Histological findings include acantholysis or lichenoid findings in Pemphigus but also lichenoid changes can be seen. Now direct immunofluorescence generally shows IgG, and sometimes IC3 intercellular areas but sometimes granular or linear deposits can be seen in the basement membrane zone.

False negatives are very common due to necrotic tissue and in early lichenoid lesions. So the serum antibodies that bind to cell surface and mucosa in a typical pattern of pemphigus can be seen on monkey esophagus and also IIF is positive in rat bladder epithelium. So multiple, auto antigens are involved in this condition. And a lot of these autoantibodies can be demonstrated using IIF and ELISA but tests are not available for many of these. And you know, we are working on bringing a comprehensive testing for this condition at our lab.

So this shows the DIF findings in Paraneoplastic Pemphigus may see very faint or weak intracellular deposits. And there are also some deposits in the basement membrane zone. So when we see something like this in the lab, I do like to mention it in the report, that this is kind of suspicious for Paraneoplastic Pemphigus, and we should try doing serum studies to rule this out.

Now, we'll talk about the serum studies that can be done. So the serological diagnosis of Paraneoplastic Pemphigus should include the demonstration of antiplakin antibodies. And these can be detected by indirect immunofluorescence on rat bladder epithelium because compared to monkey esophagus, this epithelium is richer in desmoplakins compared to desmogleins. And a positive result implies the presence of these antibodies. This test has a sensitivity which has been reported to range between 66% and 86%, and it is 83% to 99% specific. Now, we have a commercially available ELISA for Endoplakin antibodies and immunoblot for Periplakin and endoplakin is offered in research labs only and this can also be very helpful.

This endoplakin ELISA is being used in routine at our lab now, and in our experience it has a sensitivity to 75%, and a specificity of 97%. Besides doing IIF on rat bladder, and ELISA for endoplakin antibodies and indirect immunofluorescence on monkey esophagus is done for IgG, IgG1, and IgG4 subclass antibodies. As well as ELISAs for the DSG 3 and DSG1.

So this photograph here shows this reaction on rat bladder epithelium, but indirect immunofluorescence in the patients with Paraneoplastic Pemphigus, compared to a negative control, which is completely dark, this epithelium is lighting up.

This table here just shows a small study we did at Beutner Labs for ELISA, endoplakin antibodies. We tested 16 patients which were confirmed with rat bladder, IIF. And the endoplakin, ELISA. 12 of these patients, tested positive giving us a sensitivity of 75%. We had only one false positive in the Pemphigus group where we tested 10 Pemphigus patients and one showed positive antibodies. So this gave us a specificity of 90%, which is pretty good. So, now the table here just compares, I apologize for all the numbers and figures here in so much data here. But this is just to show that the tests that are available for Pemphigus and its subtypes give us over 90% sensitivity and specificity, but no single test is the answer. We have to do a combination of multiple tests to rule out this disease, to confirm this disease and to see which type the patient has has.

I'll move on to the Pemphigoid group of diseases now and I will talk about the diagnoses and also differentiation of this group. Now the subepidermal blistering diseases are characterized by autoantibodies to basement membrane components. It's a heterogeneous group and it comprises various conditions, like Bullous Pemphigoid or Mucous Membrane Pemphigoid, Ocular Pemphigoid or we have gestational pemphigoid. We have epidermolysis bullosa acquisita, the bullous lupus erythematosus, laminin 322 autoimmunity, epi-ligand autoimmunity, lamina-gamma 1 autoimmunity, linear IgA bullous autoimmunity dermatosis or dermatitis herpetiformis. So now, direct immunofluorescence is very important, as an initial confirmatory test for these diseases, because it gives us the immune deposits in the dermal epidermal junction. And depending on what Isotype we are seeing. Whether we are seeing multiple our single Isotype and the pattern of deposits we can sometimes distinguish between these two types, between these types of diseases. But serology is indicated for further differentiation.

So, here are the photomicrographs for direct immunofluorescence on skin or mucosa biopsy in pemphigoid and related conditions. So we see this characteristic linear deposition between the epidermis and the dermis. So this is the IgG and here we have IgG4 for deposition. In this figure C from mucosa and the epithelium has completely separated from the lamina propria. We can see something here on the edges of the epithelium. So this is consistent with mucus membrane pemphigoid. Then Figure D shows IgG deposits in ocular pemphigoid, and this is the conjunctiva specimen. Where we can see deposits in the junctions of epithelium and the lamina propria.

Again, here, I just wanted to show that when I initially talked about when we do direct immunofluorescence, the biopsy should always be collected away from the lesion and mucosa and never from the edge or from the lesion.

This is one example here from the same patient. The bottom two images were taken from the lesional area, completely negative. We don't see anything here, and the upper images of these

three images are from a normal biopsy, a biopsy away from the lesion. I apologize that epithelium is upside down here, but we can see this edge of the epithelium, the junctional side, lighting up, and this is IgG4 deposits in the area which is consistent with mucus membrane pemphigoid. So happy to see just as a biopsy, it would have been a negative DIF but this normal biopsy showed us that immune deposits. So that's the site of biopsy, is very important DIF studies.

So sometimes we can do direct immunofluorescence in a split biopsy. What happens in this test, this test helps us to differentiate between different forms of subepidermal bullous epidermal diseases. If our initial biopsy is completely intact, and it's positive. As we see here, we see linear IgG4 deposits and this biopsy has no microvesicles, and no separation. So we can do it directly, we can immerse this biopsy in normal saline and then take it out after 24 hours and do DIF on it again.

In this test, normal saline causes are split of the epidermis and the dermis. And we can see the deposits on the epidermal roof of the sections. So, this indicates that the patient probably has Bullous Pemphigoid, and we can confirm it by doing serology studies for BP 180 and 230. But on the other hand, if after the split, we do the DIF and we see the base of those microvesicles lighting up. It means that the patient probably has a sub-laminar densotype of sub-epidermal bullous disease like epidermis bullosa aquisita or ... or it could be laminates 332 autoimmunity or lamina gamma one autoimmunity.

44:57

And sometimes, besides deposits in the dermal epidermal junction we may observe deposits in the epidermis, as we see here. There's some little dots here in the epidermis and, in some areas. And, the other areas reveal these deposits in the junction. So this may point to a condition called Bullous Lupus Erythematosus where the patient has antibodies to type seven collagen. And these different dots could be the ANA that we are seeing in the epidermis.

So this will help us to guide the physician to do specific tests for this condition. And we have collagen 7, ELISA that are available to detect these autoantibodies.

45:42

So, sometimes if instead of IgG we start seeing IgA. It could point to the diagnosis of Linear IgA bullous dermatosis. As you see here you see this linear deposition of IgA antibodies and most likely, this patient has a condition called LABD.

46:00

The target antigens in LABD, are known as LAD97, our LAD120 and both these are a part of the BP180 molecule, which is the auto antibody in Bullous Pemphigoid. Sometimes antibodies to type 7 Collagen can be seen in this condition. This photomicrograph here shows granular deposition in the dermal epidermal junction, which is characteristic of the Dermatitis Herpetiformis.

So how do we differentiate between these different forms of sub-epidermal bullous diseases that we just looked at? So serology is an important adjunct that is needed for this differentiation. And it helps to confirm the diagnosis, and also tell us what autoreactive antigen is. And for this, again, two tasks are available: indirect immunofluorescence and ELISA. So, indirect immunofluorescence can be done on two substrates. Monkey esophagus and on normal skin and salt split skin.

So at Beutner labs, we generally, start with the IIF on monkey esophagus to see the presence or absence of basement membranes own antibodies, and, um, we report the titers of the BNZ antibodies we see and if a monkey esophagus is positive, we run tests on normal and salt-split skin.

If you see reactions on the roof, off the split skin, it points to the need for ELISA 180 and 230 antibodies, and it indicates that this patient probably has Bullous Pemphigoid.

If we see reactions on the floor of the split skin, ELISA for type 7 antibodies are done. And now we also have another test available for ruling out Laminin 332 autoantibodies. It's done with IIF on prospective cells.

So these are images from soft split skin. In Figure A, we see that the patient serum reacted with the epidermal roof. In figure B, it reacted on the Dermal floor. So if we see something like we see in Figure A, we would follow up with tests for BP 180, or BP 230. And if we see this here, then we follow up with tests for type seven collagen or for Laminin 332 autoantibodies.

I wanted to give the sensitivity and specificity data for various points of DIF for different forms of Pemphigoid and the sub-epidermal blistering conditions. We see here that the DIF has a very high sensitivity for diagnosis of these conditions and false positives are not usually seen, but false negatives are. Due to the reasons I described before. And all positive and negative cases should be followed up by serum studies. This is because it helps to confirm the diagnosis and also gives us information on the type of subepidermal blistering disease the patient has.

Again, I apologize for so much information on this in this slide, but just to say that a combination of DIF, IIF and ELISA is needed for confirming and determining what type of Pemphigoid a patient has.

And besides a diagnosis of Bullous Pemphigoid, BP180 and 230 ELISAs can be used to monitor the disease activity. And studies have shown that. High index values at baseline or when the patient is first diagnosed, can indicate a disease relapse and also indicate that the patient may have a severe disease. However, this should not be used as a standalone test for diagnosis. You have to use IIF, and DIF studies. And then BP 180 and 230 are combined together, they have over 90% sensitivity for ruling out Pemphigoid.

So, now, I'll talk a little bit about the tests that are available. And we are very thankful to the researchers who keep working on these newer techniques and making these tests more efficient and accurate.

One of the new technologies that's coming up is called the Biochip Technology, where multiple antigens are present in one well of the slide and just one test can determine what antibody the patient has. These tests are not yet approved here but I'm hopeful that they will be someday and it will really be helpful to have just one test for all the other antibodies that we need to look at.

And these are the images from that Biochips slide. And I would like to highlight this figure G, which is actually the IIF test for laminate 332 autoantibodies on transected cells. It's offered by your immune and we started running it at Beutner labs recently and it helps to rule out epiligrand auto immunity. It's done in patients that show reaction on the dermal floor of salt split by indirect immunofluorescence.

Similarly, we have multiplex ELISA. Multiple antigens are put in the well of the ELISA plate. And we can look for all these antibodies together rather than running 5 or 6 ELISA separately. In a record reported sensitivities but not yet available in most labs here in the US. So to conclude, I can say that the diagnosis of Pemphigus and Pemphigoid is made based on clinical

findings here. Serology and DIF which still remains the gold standard. but it gives limited information on target antigens. And for this serological studies for DIF and ELISA are needed to confirm and also to differentiate the circulating on the target autoantigens. I will stop here now and thank you for your patience and for listening to me and I can open the session for any questions that we may have.

Becky: Dr. Grover, it looks like you presented a lot of information for us and we really appreciate that. We have gotten some questions about your presentation. And if you could just quickly share with us. You had mentioned clinical sensitivity and specificity. Can you describe how those correlate in getting the diagnosis and what the difference between those two things are on the biopsies?

Dr. Grover: So with sensitivity we mean that if the patient has the disease, what is the likelihood that this test is picking that patient? In other words, it takes true positives and a test with the highest sensitivity is the best, but when we're trying to get a high sensitivity. The specificity sometimes is not so high. With specificity, we should have very few false positives. So if the test is specific, false positives will be very few.

Becky: Great. We've also gotten a few questions. Can we see your slide again on the proper biopsy, biopsy sites and how and where to do the biopsy?

Dr. Grover: For Pemphigus and Pemphigoid we wanted to do a biopsy from an area which has part of the lesion, maybe one third biopsies should be lesion and two thirds should be normal skin, are skin adjacent to it. This is only for skin lesions. For mouth lesions, we want to go away from the lesion at least 10 mm I would say, not even three. Anything between 3 to 10 is good because we do not want to biopsy this area here.

Becky: Great. Thank you. Another question that has come up a couple of times for us. You talked about the salts, or split skin test, that is only done on the biopsy or is that done on the blood as well?

Dr. Grover: It can be done on both, on biopsy and on blood. It can be done on a biopsy. That does not have pre existing lesions. Like, I get phone calls, a lot of times when I report positive Bullous Pemphigoid or EBA. When we see deposits in the junctional area with the DIF, we will report any of these diseases that have antibodies to the functional area or to the DNC. And to rule out we advise doing either direct immunofluorescence on a split biopsy or serum studies. But if I see that the epidermis is already separated out from the dermis or there are micro vesicles. I will not want to do a DIF on a split on that biopsy. The reason being that it can be artifactual and can meet deposits both on the epidermal roof and dermal floor and we are stuck

when we don't know what it is. So, it can be done only if it's a very normal looking area in primary direct immunofluorescence.

Becky: Great, thank you. We also got a question that asked, If a DIF or IIF can be done if a patient is in remission or do they need to have active lesions? I know that some of these have overlapping biopsy sites and others are away so will it still show up even if you're in remission or don't have any active lesions at the time?

Dr. Grover: So, in direct immunofluorescence and ELISA can be positive in patients in remission. And direct, theoretically, DIF can also be, but I don't think any physician uses DIF to monitor disease activity, obviously because you know it's more invasive, but serology is used to monitor disease activity, but yes, we can have high index values for ELISA's in patients in remission and also IIF can.

Becky: Great, thank you. We also got a question from Jane. She says that she's had three biopsies, the histology was done, suggested Cicatricial Pemphigoid. She's had two direct immunofluorescence biopsies. One showed no results, and another one showed that it wasn't an inadequate specimen. But she does have erosive, lichen planus in areas of her body. How do doctors in the lab and the lab determine if it's the erosive lichen planus and not Pemphigoid?

Dr. Grover: Yes, I should have included a micrograph of that. So, then we do the DIF in mouth regions. That's why we advise doing two biopsies. Biopsy from a normal area to see any deposits, suggestive of mucous membrane pemphigoid and a lesional area is needed for. And in lichen planus we will see a very characteristic shaggy deposit and also fibrin. And I've seen patients where you see both the findings in biopsies from different areas, like if the collective biopsy from normal area, we will see findings suggestive of cicatricial pemphigoid. And if we take a biopsy of a lesional area, we will see erosive lichen planus changes. And these changes actually can go together. Patients with erosive lichen planus can then start developing a cicatricial pemphigoid. So in her situation, I'm sorry that she had to undergo two labs with no confirmation yet. But maybe the lesions in the mouth are very friable and for DIF it's very important that we have intact epithelium, and underlying tissue when we want to look at those deposits. So maybe the area that was biopsied was very inflamed, and it was an older lesion. You can try testing again on a normal area, and also sometimes adding IgG4 tests is helpful. Like, we may not see IgG, but G4 shows up very strongly. At least shows up when IgG are not conclusive.

Becky: Great, thank you for that. Morgan asks, Is there a difference in what you see in pathology with patients who have been diagnosed after testing positive for Covid 19

Dr. Grove: I'm sorry, we don't have this data here. And when we got specimens, we generally, I'm not told that the patient has had Covid 19 or not. But we have had positive Bullous Pemphigoid after Covid. So, the patient had Covid, and within two weeks started developing skin lesions and it turned out to be Bullous Pemphigoid. Is it coincidental or is it causing it? We don't know.

Becky: Ok, also, you had mentioned during the presentation about false negatives. Are there such a thing as false positives in doing the biopsies or the blood work?

Dr. Grover: So, usually, no. If in an experienced lab that has experienced reading those biopsies, I would say no. The only thing that I've started noticing recently is that sometimes biopsies have a very strong non diagnostic fluorescence everywhere, you know, in the cell surface, which can actually look like pemphigus and I myself have gotten doubtful. But then when we run the IgG4 test, that helps us to rule out and you can always conform with serum studies. So if simultaneous serum studies are done, IIF is negative, DSG 1 and 3 are negative, you can pretty much rule out these false negatives, false positive cases.

Becky: Great. I learned a lot this hour from you Dr. Grover, and I really appreciate you being here and sharing all of your knowledge and your expertise with our community. I hope everyone who listened in with us today was able to glean some sort of information from this. I know it was a pretty technical topic today. But I think this is a very good way to dive into what these biopsies are, and what the blood work is.

I would also like to give a huge thank you to everyone on the call for joining us today and thank you to Sanofi Regeneron Genentech, and argenx, for helping to make today's call possible.

Before we go, I have a few announcements, I am excited to announce that this year's Patient Education conference will be held virtually from October 21st to October 23rd! We've invited leading bullous disease experts to present on research and trends, educate on disease management, and answer your tough questions. The 2022 Virtual Patient Education Conference will be an exciting and educational event for any patient, caregiver, physician, researcher, or stakeholder in the field of bullous disease. Registration will be opening soon and we hope you will join us for this exciting event.

Do you wish there was a better understanding of our diseases by doctors and researchers? Do you wish there were more FDA-approved treatments and better treatments available? Well here's your chance to get involved and make these goals a reality - Join the IPPF Natural History Study today!

The Natural History Study is a patient registry sponsored by the National Organization for Rare Disorders (NORD) and the US Food and Drug Administration (FDA). Your information is

private, the IPPF Natural History Study follows strict government guidelines to assure patient information is protected. Your participation and the data will be used by the IPPF to help advance research, better understand the patient journey, find better treatments, and hopefully one day a cure. By sharing your journey and answering some questions, you directly have an effect on the future of all people affected by pemphigus and pemphigoid. So get involved today! You can find the Natural History Study by visiting www.pemphigus.iamrare.org

Do you want to become a hero in our community and continue to support the free services the IPPF provides to you, such as today's webinar, the IPPF's Peer Coaches and our find a doctor map? If so, become a Healing Hero today! Healing Heroes fund the future of the IPPF community by making sustaining, monthly gifts to support our mission of improving the quality of life for all those affected by pemphigus and pemphigoid. No amount is too small even a \$10 or \$15 monthly donation goes a long way and continues to allow us to provide for the greater good of our community.

The IPPF has a number of upcoming virtual support groups across the country. If you are interested in attending a meeting, please check the IPPF's Event Page to register for a meeting. Also, we are always looking to expand our support network. If you are interested in starting a support group in your region please contact me, Becky Strong at becky@pemphigus.org. It's easier than it sounds to start a support group and you can help connect others in your area with other patients.

This call recording will be sent out with the survey following this call. Thank you all for joining us.