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Digital quantification of lymphocytes in breast cancer

Dr Hartman: Hello, I am Johan Hartman, I am Professor of Pathology at Karolinska Institute. And we will talk together with Balazs Acs about digital quantification of lymphocytes in breast cancer. And we are working at the Karolinska Institute in the Karolinska University Hospital in Stockholm, Sweden. So, these are disclosures. So, we will start, I will start briefly by talking about TILs tumor-infiltrating lymphocytes, the importance in breast cancer of TILs, and also, where we stand in terms of the current diagnostic area and research. Then, Dr Acs will proceed and talk about digital access TILs and the workflow, how we can do this in an open-source platform. In the end, also we will conclude and understand with the digitally assessed biomarkers where we... what we still need to develop. So, tumor-infiltrating lymphocytes actually means in clinical practice the way how we measure anti-tumor immunity. So, in tumor-infiltrating lymphocytes occur in many different areas of solid cancers, but in breast cancer we are mainly interested on the stromal TILs, the tumor-infiltrating lymphocytes, the mononuclear immune cells that occurs within the boundary of the tumor which means not outside of the tumor, but inside the tumor, but in the stroma between the tumor cells, the islands of tumor cells within the stroma. You report the stromal TILs as percentage value, which means the percentage of stromals area occupied by these mononuclear inflammatory cells. And it's important to understand that this assessment is made on a routine stained hematoxylin-stained images slides, not by immune histochemistry. So, why are tumor-infiltrating lymphocytes important in breast cancer? So, in the figure here to the left, you can see the different intrinsic molecular subtypes of breast cancer. This is one of the most important ways how to classify breast cancers. And what we see here is the ER-positive luminal subtypes, they have an outcome that is most often superior compared to the triple-negatives and HER2-positive breast cancers. Between these different ER-positive you have a difference in the Luminal A and B, which mainly is the difference in proliferation and aggressiveness. So, the Luminal B subtype of cancers have a much poor outcome. So, so far, we understand that the tumor-infiltrating lymphocytes might have different roles in ER-positive cancers, the luminals compared to the triple-negative and the HER2-positive. And we have most compelling evidence from the triple-negative breast cancers, and as you can see in the figure to the right, within the triple-negative breast cancer subtypes, which is somewhere around 10% of all breast cancers, we can see that irrespectively here of number of axillary lymph node metastasis, there is a prognostic role of tumor-infiltrating lymphocytes. Here we have the different boundaries, the thresholds, what is high TILs and what is low TILs might differ in some different studies. Here we have a boundary of 30%, what we can see is

that the triple-negative breast cancers with more than 30% of tumor-infiltrating lymphocytes, have a much better outcome compared to the ones with less tumor-infiltrating lymphocytes. So, and what is important here also to add here is that the data is most compelling, most strong in the triple-negatives, but also in the HER2-positive subgroup, we are starting to get important data to consider the TILs also in this subtype, where also TILs appear to be prognostic. So, there have been, of course, there are different, have been during the years, different ways how to measure tumor-infiltrating lymphocytes, and here I think there've been very important standardized efforts by the International TIL Working Group. And here we know that the TILs from these studies should include or should be measured within, as I said, within the invasive margins of the tumor. All mononuclear cells, which means also some other cells but mostly lymphocytes, should be scored, but we should exclude polymorphonuclear leukocytes such as granulocytes. TILs outside of the invasive tumor should be excluded, but also TILs in or without surrounding DCIS and the normal mammary gland structures should be excluded. We should also exclude any kind of measurements within artifacts and within necrotic or fibrotic areas. So, we should also understand that the TILs should be a general assessment, a full assessment, and not focusing on any hotspot regions of tumor-infiltrating lymphocytes. So, this is more complicated, actually than it can look like, and this is a pretty time-consuming task to do this, especially if you're doing it on all cases. Of course, it's different if you do it on a preoperative biopsy compared to a resected specimen, but even a preoperative biopsy takes time to do this carefully. This is an example here of different regions in two different tumors that it might also, and what is very common is that there is heterogeneity in your inflammatory patterns, that there might be some areas with more tumor-infiltrating lymphocytes and other areas with less. So, in the end, you need to consider here the general measurement of TILs. This has also been clearly showed here by some authors. This is... was one paper, a study by Carsten Denkert and colleagues, showing you that there's a huge variety and a poor, actually pretty poor, reproducibility if you look at the specific levels of TILs in breast cancers, where you can see that this really calls for additional decision support tools and systems for the pathologist to make a more reproducible and accurate assessment of TILs, since TILs now, as we understand, have an important prognostic role in triple-negatives and also in HER2-positive breast cancers. By that, I hand over to Dr Acs.

Dr Acs: Thank you very much, Dr Hartman. I will step in and continue the presentation focusing on digital image analysis with an open-source platform name QuPath. So, might be known that QuPath, developed by Peter Bankhead by that time at the Queen's University Belfast. And it’s open source, allow to write extensions and scripts to score a variety of biomarkers in the field of pathology, but also research wise. And it supports almost all image formats, most importantly all the whole slide image formats from different vendors of scanners, but also the usual ImageJ or JPEG formats. And the basic workflow, what I would like to show here today is the estimate stain vectors, cell segmentation, classification training and getting the results in QuPath. This is a very basic workflow, maybe you can develop your own system which suits you better, but let's see in the details. So, the estimate stain vector commands here you can see a representative image of an H&E slide. And the yellow rectangle shows an area, and this is the visual stain editor showing each pixel with the representative colors showing for each colors, I mean green, red, and blue. And the lines showing the current thresholds or the default thresholds for these values to distinguish these colors. And you can see many of these pixels or spots are out of these thresholds, so one should give and find the estimate stain vectors on your study stainings. And by doing that you can just click the auto button and the software finds the best thresholds on your representative area, and this is very important that you have to find a really representative area showing the ideal staining, so, try to avoid artifacts. But, of course, you can find more information on QuPath website about this. And I just want to show you how does it work on the, for example, Eosin. You can see that there is a bunch of Eosin stainings in the cell nuclei, which is not ideal for cell segmentation. And after this estimate stain vectors, you can see that the Eosin diminished from the nuclei. So, let's move on to cell segmentation. QuPath uses an unsupervised watershed cell detection, where you can set the settings, the parameters for cell detection for nuclei, how big nuclei you wish to detect, what's the minimum area, what's the settings, would you like to set to separate the nuclei and the threshold for

intensity. And then, we will see an example with the default values, which is always a good point to start, to detect all the cells in QuPath, its objects. And then, we move on to the classification and training. You can set up your own algorithm. You should, you can classify tumor cells, immune cells, stromal cells or different population of objects. Try to annotate as much cells and homogeneous cells for each class, I mean, not mixing up with different cells for annotation. It should represent a heterogeneous tissue pattern if you want to use this generally, and actually, QuPath offers a different variety of machine learning methods such as Random trees, Neural Network or Decision trees. And then, it will give you the results for the whole region of interest that you annotated. Here's some examples of annotations from this representative image. And then, the cell classification, I mean, the cell detection will turn into cell classification representing, let's say, tumor cells, stroma and lymphocytes. And here's actually the workflow from our study published recently showing to, or aim for build up a TILs scoring method in triple-negative breast cancer. And we just use the same pipeline that I just showed you here, so, basically estimate or first, define the tumor region by the pathologist, then estimate the stain vector, then move to cell segmentation and then applying the classifier. For the training of the classifier, we used a cohort for the training set and the trained the classification on the set of slides. And, of course, pathologists reviewed it and made corrections to get it better and better. And once the performance of this classifier was satisfactory, it has been locked down and it's been applied independently on different study sets. Here, you see some examples of cell detection and classification from the study. Red refers to tumor cells, purple refers to lymphocytes and green refers to stroma. And regarding the variables, so as Dr Hartman mentioned, TILs should be scored as the percentage of area they occupied within the tumor. However, with digital TIL assessment, we are also able to measure different TIL variables such as the percentage of, or the number of cells within the tumor area compared to tumor cells, fibroblasts, and lymphocytes. And, of course, but we can also analyze the whole tumor area as percentage with lymphocytes in it. And we tested all these digital biomarkers in the study groups. And this is the prognostic potential with our Digital TILs variables and what we can see that the TIL digitally measured TIL variables are prognostic in all independent study sets such as the pathologist-read and TIL scoring. And the total number of patients, of triple-negative patients, was more than 700 in this study set. Then, we also tested how the machine-read TIL scores and the pathologist-read TIL scores correlate, and although they reached a significant correlation, in clinically it doesn't mean such a nice agreement between the two, but if we just remember to the introduction, it's very hard to score TILs and actually it's not exactly the same variable as we see in digitally, and many other studies showed that the perfect agreement cannot be reached. And this is, A and B are measuring digital TIL scoring with pathologist-read TIL scoring, but the pathologists were different in these study sets, so, this is not related only to one human. And this is the study published in Clinical Cancer Research where you can deepen your interest in the methods, and even you can also download the algorithm too and with a nice guideline how to do it on your own. And of course, this is free to use, even the algorithm. And finally, I would like to conclude about digitally assessed biomarkers here in this case TILs, what we need to still learn about this. So, important question, what is the ground truth? It should be the pathologist-read score? As we just saw from Dr Hartman, the pathologist scores can be variable and may or may not reach a high concordance. Should it be then the outcome as the ground truth? I wouldn't think it should be so simple that we rely only on outcome defining the biomarkers. Then the next question is how much discrepancy between digital tools or between pathologist and digital tools are clinically acceptable? So, even if we see a difference between pathologists or between different digital tools, is it clinically relevant? So, this is an also important question to address in the future. And then which cut-off we shall use, or used as continuous variable for clinical practice? Actually, this is suggested by the clinical, the TIL Working Group, that TILs should be added as a continuous variable in the prognostic mammograms and not as a binary valuable with the specific cutoffs. And this is also important that we have unfortunately, a lack of publicly available annotated data sets to aid the scientific community to validate such important computer tools, scoring any biomarkers including TILs. And yes, the lack of reference materials that can be used to compare different computational tools, this is also very important, and I think many Working Groups is working on this to address this important question, these should be comparable tools. And, of course, important question rise that should

digital tool be validated for different tumor subtypes? For example, in breast cancer, we have different histological subtypes, and shall we use different algorithms for these subtypes, because TILs doesn't look different, although tumor cells might be look different. Or even this digital tool should be validated on different specimen types such as core biopsies or surgical specimens. So, these are the important questions that should be addressed in the future. And I would like to thank you for your attention, and we are happy to take questions. Thank you very much.