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## Digital pathology biomarkers for breast cancer

**Prof Laurinavicius:** I thank the organizers for this opportunity to present on digital pathology biomarkers for breast cancer and, you know that pathology's slides and grading contain enormous information about pathology and tumor pathology. Since we can digitize those slides, we can apply many analytical tools, mathematics, and implicit deep learning modeling to extract more and more sub-visual information. In my presentation, I'm going to talk about mainly image-based computational biomarkers. Which actually, the digital biomarkers, what we mean by this concept, which our team has developed to assess Intratumor Heterogeneity and Tumor-Host Interaction in the Tumor-Host Microenvironment. Then, I will show that these two concepts can be combined in prognostic models. Let's start with the Intratumor Heterogeneity. The concept is well-known to oncologists and pathologists. No need to dwell on this. And then we have the concept of hotspots quite commonly used in pathology. If we look at real-life pathology, what pathologists do to assess, for example, proliferation rate in breast cancer. You know this is one of the typical examples of the Intratumor Heterogeneity problem. So, what pathologists do, they apply their visual assessment with a microscope. You can think of ir as a tube-vision, then we assess semi quantitatively the areas we generate numbers in our brain. Then we maybe detect the hotspot. We go there and count cells, which, as you know, is quite time-consuming. Instead of that, we thought, what if we just run this assessment systematically, we generate local proliferation rates, and then, we see what to do with the data. And actually, what we did, we applied hexagonal grid to compute, to assess the local professional rates in each area of the tumor. So, actually, we process the data obtained by image analysis, it was over six years ago. And the reason why we choose this hexagonal grid, it's a common question why it is better than a square grid, for example, that is pretty simple, it's the best geometry to cover the area invented by bees and that's common in nature. So, mathematically, it allows you to compute local neighborhood transformations, or changes in three directions and without much bias. So, that's the general idea why it is better. Then, it allows you to compute features which you see in the next slide here. Just from a single image analysis data on Ki67 you can achieve quite a comprehensive list of indicators, which represent both sample size, density, cellularilarity, proliferation rates - in many expressions, means, median, percentiles. You can also set bimodality in the data sets. And importantly, there are indicators called texture indicators proposed by Haralick. For example, Haralick's spatial entropy allows you to assess how disarranged is the biomarker expression is within the tumor section. It just gives you one quantitative measure of the Intratumor Heterogeneity of any biomarker. Also, you can assess bimodality and the system can show a map of the areas of the tumor where the second spike is. You can also visualize this to have a feeling of "roughness" of the tumor surface in terms of a biomarker expression. We tested this approach on an independent cohort of Nottingham breast cancer patients. With the hypothesis that maybe a hotspot, as we suggested, the Pareto hotspot, just taking the 10th percentile of the proliferation rate would be the best way to measure this. But by surprise, we found that bimodality measured by Ashman's D indicator was the best independent prognostic indicator of patient survival. The patients with high bimodality, presented with worse survival probability. But what I will show you later, we

have more data revealing that actually, if you measure heterogeneity - you are able to quantify it, it is more informative than measuring the proliferation level, even if you would try to detect hotspots, with visual, defined by pathologist, by the way there are not agreed definitions for that, or even machine-generated hotspots do not collect the systematic measure of the Intratumor Heterogeneity. Our second model was dedicated to assess Tumor-Host Interaction. And here, we proposed two new methods. Both these concepts I will explain in a moment, to measure Immunogradient at the Interface Zone of tumor and stroma. Why it is important? Mainly, it's becoming very important with the advent of modern immunotherapy modes. It has been shown that tumor infiltrating lymphocytes and their spatial distributions are very informative. This is just to illustrate which is pretty well-known for pathologists and oncologists, that compared to the chemotherapy or even targeted therapy, you have a huge benefit in terms of long-term patient survival in many cancers. Pioneers, who invented and actually applied to a measurement of infiltrating lymphocyte distributions in colorectal cancer, called it Immunoscore, and as they recently reviewed the literature on that, they made a statement that half of the information in colorectal cancer is still underutilized and we need to measure host-immune response in many ways, it maybe of high prognostic and even predictive value in many solid tumors. There are many methods proposed to assess the spatiial aspects of host immune / inflammatory cells attacking tumor cells. Some of them are measured by the low distances between tumor cells and different types of lymphocytes. Some methods propose clustering, detect clusters' location, and then even highplex-based methods to allow very sophisticated, indicators to be extracted from the tumor. Our own approach was again, based on the hexagonal grids. So, we asked where this tumor enters the interface, is it just a line, a ribbon? Is it commonly to used many methods. Or, this is more flexible, more intricated area of tumor-host interaction. So, we asked if we can develop a set of rules to just say where is high probability of tumor-stroma meeting each other. This method is just briefly showing the workflow that is based on actually any immunohistochemistry, but in this study, we used CD8 immunohistochemistry, scanned, applied the HALO AI tool for tumor-stroma segmentation and then quantified CD8 positive lymphocytes. And here is the method which I will explain here in the next slide. So, what it actually does, the first step is to assess the probability of the “interface-ness”, as we call it, to say where's the tumor edge, which is depicted here as yellow hexagons. Actually, it is based on the contents of this hexagon, then it assesses the contents of the tumor and stroma around, and in the second-generation, and then, it computes in different directions. That's pretty sophisticated formula, but it works pretty well. And then, what we'll do next, we define where we have stroma aspect, and then we go deep into tumors, so, we can rank the hexagons towards the tumor and towards the stroma, which is depicted here as different shades of green and different shades of red. This spatial information allows us to compute density profiles within this interface zone and showing the trend of lymphocytes to “move” towards the tumor. Sometimes they are increasing, sometimes they just stop here at the tumor's edge. For quantification of these features, we tested many formulas, but we found that center of mass computed from these ranks and another indicator, which we called ImmunoDrop - meaning the ratio between the rank minus one in the stroma and rank plus one, in the tumour. So, it depicts a sudden drop just passing the tumor edge. Here is an example of breast cancer - surgical excision sample where we have this automated tumor and stroma interface zone extracted. Importantly, we sample this area automatically, and this is the most strategic area where tumor and host interact. Then we compute the Immunogradient. We developed this method in parallel in hormone receptor positive cancer and colorectal cancer. Actually, we obtained very similar results. I'm showing here, multiple Cox regression. It reveals that in both patient cohorts high immunogradient would have an independent impact on better patient survival. Both the direction, the gradient, and the mean absolute density of the lymphocytes in the tumor aspect, are important. Then we can aggregate this into a single factor which predicts a better patients' survival. If we look more closely here at the breast cancer patients and colorectal cancer, they have similar survival stratifications. I just want to point one interesting finding, here, if we use the Immunogradient as the stratification factor, we are able to find quite interesting phenomenon. This is the hormone receptor positive breast cancer. You can tell it's a well-managed disease: after five years of surgery, 92% of the patients are alive. But then, what happens in the next five years? We have quite a dramatic divergence from these probabilities, meaning that in the

patients with low immunogradient detected at the point of surgery have 55% survival probability compared to 87% after 10 years. So, it reveals that by measuring the lymphocyte response in this way, we are able to predict long-term survival. Maybe, it also may have clinical implications requiring more comprehensive management of these patients long-term, and maybe immunotherapy options. Another patient cohort, which was published just last week, in November. Again, these are hormone receptor-positive breast cancer patients, HER2-borderline by immunohistochemistry, but not amplified. You might think it's HER2-enriched, hormone receptor positive cancer. There was another interesting finding. From many prognostic models tested we found that immune response, again, measuring CD8 Immunogradient was most important. But what is new here, that in addition to the directionality and to the absolute density on the tumor aspect of the interface zone, we have standard deviation of CD8 density along the tumour edge acting in the opposite direction - as a feature of worse prognosis, and all three computational biomarkers present independent contribution for prognostic modeling, which is illustrated here by combining these three features into a single score, you have quite strong patients' survival stratification. So, what is new here, that we found that this interface variance of the density or irregularity of the TILs around the tumor discloses worse prognosis in these patients; even if there are lymphocytes, it's important to follow along the zone. So, actually what it goes to show that we are able to get three independent prognostic image-based biomarkers from a single CD8 slide. So, in some way, we have computational augmentation of the data. Can we combine these approaches which utilize both heterogeneity and interface and gradient features? This was another study where we used six biomarkers, in hormone receptor positive breast cancer. At the end, we found that independent prognostic value was detected by these three indicators. I will show on the next slide. But what is again important that we were able to predict patient survival exclusively based on immunohistochemistry. No conventional clinical or pathology features were selected by the models. Again, we find that high proliferation bimodality predicts worse prognosis; again, we find that a specific subset of CD8 with SATB1 expression (which is a feature of epigenetic regulation in active lymphocytes), but the surprise comes also with this finding - high progesterone receptor entropy was the feature of better survival. Puzzled by this finding, we looked more closely at the distribution of the progesterone receptor. So, we have a plotted here of the entropy - disarrangement with the progesterone receptor against its expression level within the tumour. We find high entropy in the middle range of the expression, we have the survival here, which can also converged in these survival curves. It is hard to explain this. It requires further investigation, to understand this phenomenon, but we will look back at the knowledge of what we know on the role of hormone receptors. There are many studies performed, 19 studies were recently reviewed involving 30,000 patients. The conclusion was that there was no clear evidence for quantitatively assessed hormone receptor prognostic or predictive value and maybe our finding explains why many previous studies have contradictory results because they have different assessment and quantification methods. But they also, where not investigating tumor heterogeneity and some non-linearity aspects which may be relevant in the biological behavior of these cancers. So, to summarize what we were getting from our studies is that we find that spatial intratumor heterogeneity of biomarkers, Ki67, progesterone receptor, are more informative than just the level of the biomarker expression. We also showed that we are able to measure directionality, spatial directionality at the most strategic place of the tumor and stroma interaction. We get better models to predict patients' survival, and finally, it is possible and it's getting pretty common to get exclusively immunohistochemistry-based models to perform better than conventional parameters currently used in clinical practice. I would also address to this recent interview which we published this year. It shows the roadmap, the perspective of what is actually happening with these digital computational and image-based biomarkers. We have the question to ask, focused towards immunohistochemistry or any other technique. Then we have a broad set of tissue processing techniques available. Then we have various imaging techniques and then we have various computing and machine-learning methods to apply, to get the useful clinical information, diagnostic, prognostic, or predictive. There are many, many opportunities on the path to go. I would like to acknowledge our research team here and also, our collaborators in France from Normandy University and also, on this project, which is about to end granted by the European Social Fund. Also,

collaborators from the United States, Michael Shribak of the Marine Biological Laboratory, Kuang-Yu Jen and Richard Levenson, University of California Davis Medical Center. Thank you for your attention.