

Reference Material for the Assessment of Stromal Tumor-Infiltrating Lymphocytes in the High Throughput Truthing Project

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Table of Contents

Section	Page
I. Executive Summary	3
II. Introduction	4
III. Performing the sTILs Assessment	6
IV. Pitfalls in the sTILs Assessment	7
V. Clinical Pearls for Approaching sTILs Assessment	19
VI. Annotations and Discussions	24
VII. References	79

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I. Executive Summary

Tumor-infiltrating lymphocytes (TILs) have been identified as prognostic and predictive biomarkers in various cancers. Among breast cancers, those found to be estrogen, progesterone, and human epidermal growth receptor 2 negative, known as triple negative breast cancer (TNBC), have stromal TILs (sTILs) densities that correlate with patient outcomes [1–8]. Understanding this relevance, incorporating the TILs assessment into standard clinical practice is endorsed by international clinical and pathology organizations [9–11]. This document serves as reference material for the sTILs assessment of TNBC as part of the High Throughput Truthing (HTT) project, with the sTILs assessment outlined in accordance with the guidelines developed by the International Immuno-Oncology Biomarker Working Group (the Working Group) on Breast Cancer [12,13]. In this document, we begin with an introduction to the HTT project and collected annotations for the sTILs assessment. The introduction is preceded by a summary of the sTILs assessment, sTILs assessment pitfalls encountered in the dataset (with examples), and clinical pearls from our expert panel. It concludes with an image of each region of interest encountered in the training batch of the dataset and the corresponding expert panel annotations, commentary, and pitfalls. This document has been prepared in conjunction with the Garcia et al. manuscript titled “Development of Training Materials for Pathologists to Provide Machine Learning Validation Data of Tumor-Infiltrating Lymphocytes in Breast Cancer” published in the Cancers Special Issue “Tumor Infiltrating Lymphocytes (TILs) in Solid Tumors: Emerging Insights” (<https://doi.org/10.3390/cancers14102467>) [14]. Portions of this document’s text are recapitulations of the manuscript and adapted for training in the clinical assessment. Please refer to the manuscript for more description of this work.

II. Introduction

Tumor-infiltrating lymphocytes (TILs) are prognostic and predictive biomarkers in triple negative breast cancer (TNBC) [1–4,7,8]. The density of TILs in primary tumor specimens of patients that do or do not receive (neo)adjuvant chemotherapy demonstrate positive correlations with patient outcomes [7,8,24–26]. Thus, incorporating the TILs assessment into standard clinical practice is actively endorsed by international clinical and pathology organizations [9–11]. Guidelines for standardized TILs assessment and educational materials to support researchers and pathologists to score this biomarker have been developed by the International Immuno-Oncology Biomarker Working Group (the Working Group) on Breast Cancer [12,13].

In 2019, we began the High Throughput Truthing (HTT) project with the goal of developing a dataset for the evaluation of artificial intelligence and machine learning models that estimate the density of stromal tumor infiltrating lymphocytes (sTILs) in hematoxylin and eosin (H&E) stained TNBC breast cancer biopsy specimens. Our goal is to produce such a dataset that would be fit for a regulatory purpose as a medical device development tool (MDDT) [15][16]. The pilot study produced a total of 7,373 sTILs density estimates for 640 unique regions of interest (ROIs). The ROIs are from 64 H&E stained slides of breast cancer biopsies; a collaborating pathologist selected ten unique ROIs of varying morphology from each slide according to the protocol described by Dudgeon et al [16]. Pathologists could use optical or digital modalities: a light microscope system (eeDAP [17,18]) and two digital whole slide image viewing and annotation platforms (caMicroscope [19] and Path Presenter [20]).

By the end of the study, we finalized the protocol to collect three data elements for each ROI: **ROI type, percent tumor-associated stroma, and sTILs density.**

The **ROI type** is a qualitative variable that describes the tissue within the ROI either as “Evaluable for sTILs” (“Evaluable”) or “Not Evaluable for sTILs” (“Not Evaluable”). “Evaluable” tissues are regions where invasive tumor, tumor-associated stroma, and sTILs could be found, understanding that not all tumor-associated stroma contains sTILs. “Not Evaluable” tissues are those where there is no invasive tumor, and by definition, no tumor-associated stroma or sTILs. Given these definitions, the ROI type offers an additional opportunity to evaluate whether an algorithm is estimating the sTILs density in the proper regions.

The variable **Percent Tumor-Associated Stroma** is the percent of tumor-associated stroma present within the ROI and is calculated as

$$\text{Percent Tumor-Associated Stroma} = \left(\frac{\text{Area of Tumor-Associated Stroma}}{\text{Area of Entire ROI}} \right) \times 100\%.$$

This variable represents the visually estimated percent of the entire ROI (including empty space) occupied by tumor-associated stroma, the compartment in which the sTILs density is evaluated. For this project and dataset, the area of the entire ROI includes empty space in addition to tissue. While the percent of tumor-associated stroma is not reported clinically, appropriately identifying stroma for evaluation is a critical step in estimating the sTILs density. As such, we ask for the percent of tumor-associated stroma to remind the pathologist about the AI/ML model’s segmentation step. The data can also be used to assess an AI/ML model’s ability to identify tumor-associated stroma, a component of the sTILs density.

The **sTILs density** is the percent of tumor-infiltrating lymphocytes (TILs) area within tumor-associated stroma and is calculated as

$$\text{sTILs Density} = \left(\frac{\text{Area of Tumor-Infiltrating Lymphocytes}}{\text{Area of Tumor-Associated Stroma}} \right) \times 100\%.$$

The sTILs density and percent tumor-associated stroma assessments are quantitative biomarkers ranging from 0% - 100%. TILs are limited to lymphocytes and plasma cells. Granulocytes (e.g. neutrophil, eosinophil, basophil), dendritic cells, and macrophages are not considered in the quantitative assessment [12].

In analyzing the pilot study data, we observed notable pathologist variability in sTILs estimates [14,21]. To understand and address this variability, we established an expert panel to review a subset of ROIs [14]. When selecting the ROIs for the expert session, we only included ROIs that were determined evaluable by at least two pilot-study pathologists. We also stratified our sampling according to three sTILs density bins: low infiltration = "10% or less," moderate infiltration = "greater than 10% to 40%," and high infiltration = "greater than 40%." These thresholds and bins were suggested by our clinical experts to provide possible categories for patient management [22,23]. Currently, there are no established guidelines for the thresholds and bins. We then selected cases from within each density bin with the highest and lowest variance and entropy using a 2:1 high-low ratio, yielding a total of 72 ROIs. We organized eight recorded, one-hour virtual sessions where expert panel members discussed each selected ROI regarding their sTILs assessment.

In this document, we have compiled our Expert Panel's estimates of the percent tumor-associated stroma and sTILs density. We have also compiled their comments on features that confound the sTILs assessment.

III. Performing the sTILs Assessment

We focus our efforts on the stromal TILs assessment in accordance with the recommendations from the Working Group [12]. The TILs assessment requires preserved tissue, either core biopsies prior to neoadjuvant therapy or full sections, and is applicable to both primary and metastatic solid tumors [4,12]. The TILs assessment can be performed in both the stromal and intratumoral (also called intra-epithelial) tissue compartments. However, when using H&E-stained sections of invasive breast carcinoma, intratumoral TILs are more heterogenous and difficult to observe without additional staining. In addition, stromal TILs measurements provide the same information as intratumoral TILs while being a more reproducible measurement [12]. We prioritize core biopsies of the primary tumor, as metastatic disease is an area of current research [4,27,28].

“The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014” by Salgado, et al., offers detailed recommendations for the TILs assessment in their Table 2 [12].

Instructions for the sTILs assessment specific to our HTT project’s ROI-based approach are below:

1. Begin by examining the entire WSI and assessing the tissue and tumor architecture.
2. Return to the ROI and determine if this ROI should be assessed for sTILs. If invasive tumor and tumor-associated stroma are present, the ROI is considered “Evaluable.” Otherwise, the ROI is “Not Evaluable,” and no further action is required for that ROI.
3. If “Evaluable,” determine the percentage of tumor-associated stroma; see Equation 1 above. The numerator is the area of tumor-associated stroma, and the denominator is the entire ROI area.
4. Determine the sTILs density within the area of tumor-associated stroma; see Equation 2 above. Neutrophils, eosinophils, basophils, histiocytes/macrophages, and lymphoid aggregates (tertiary lymphoid structures) are not considered TILs for this assessment.

IV. Pitfalls in the sTILs Assessment

Our expert panel identified various pitfalls in the sTILs assessment, which can affect estimating either the percentage of tumor-associated stroma or sTILs density. The etiologies of these pitfalls include the tissue section preparation process, limitations of hematoxylin and eosin staining, and challenges in visual assessment. Table 1 provides a summary of the pitfalls; it is a copy of Table 6 in Garcia et al. A detailed breakdown and description of the pitfalls are described below with examples. For additional reading on pitfalls in the sTILs assessment, see *Pitfalls in assessing stromal tumor infiltrating lymphocytes (sTILs) in breast cancer* [Zuzana Kos, et al., npj Breast Cancer (2020)6:17; <https://doi.org/10.1038/s41523-020-0156-0>].

Table 1: Summary of pitfalls encountered during the sTILs assessment grouped by pitfall type. This is a copy of Table 6 in Garcia et al.

Pitfall Type	Pitfall Summary
Percent of Tumor-Associated Stroma	<ol style="list-style-type: none"> 1. Exclude thick-walled vessels, benign glandular elements, adipocytes, carcinoma in situ, and necrosis from the area of tumor-associated stroma 2. Calculate with respect to the entire ROI area 3. Variations in tumor cell morphology can make it difficult to distinguish stroma from tumor
sTILs Density Score	<ol style="list-style-type: none"> 1. Cells with small/pyknotic nuclei and/or perinuclear clearing can be difficult to categorize 2. Non-lymphoid cells may be confused for lymphocytes 3. Error in the percent tumor-associated stroma can affect the sTILs density 4. Sparsely distributed tumor cells may be more challenging to quantitate

Percent of Tumor-Associated Stroma

1. Not all mesenchymal tissue should be considered as tumor-associated stroma.

For the purposes of the sTILs assessment, tumor-associated stroma is defined as the reactive stroma composed of fibroblasts, newly formed vessels, collagenous and/or elastotic fibers and extracellular matrix surrounding invasive carcinoma cells. Listed below are pre-existing normal structures, such as thick-walled vessels, benign glandular elements, adipocytes, carcinoma in situ, and necrosis, that are excluded from the area segmented as tumor-associated stroma.

Adipose Tissue

Adipocytes are not considered part of the tumor-associated stroma for purposes of sTILs assessment. In cases where collagenous stromal fibers are seen infiltrating through areas of adipocytes, these stromal areas between adipocytes are considered as tumor-associated stroma, while the adipocytes themselves are excluded.

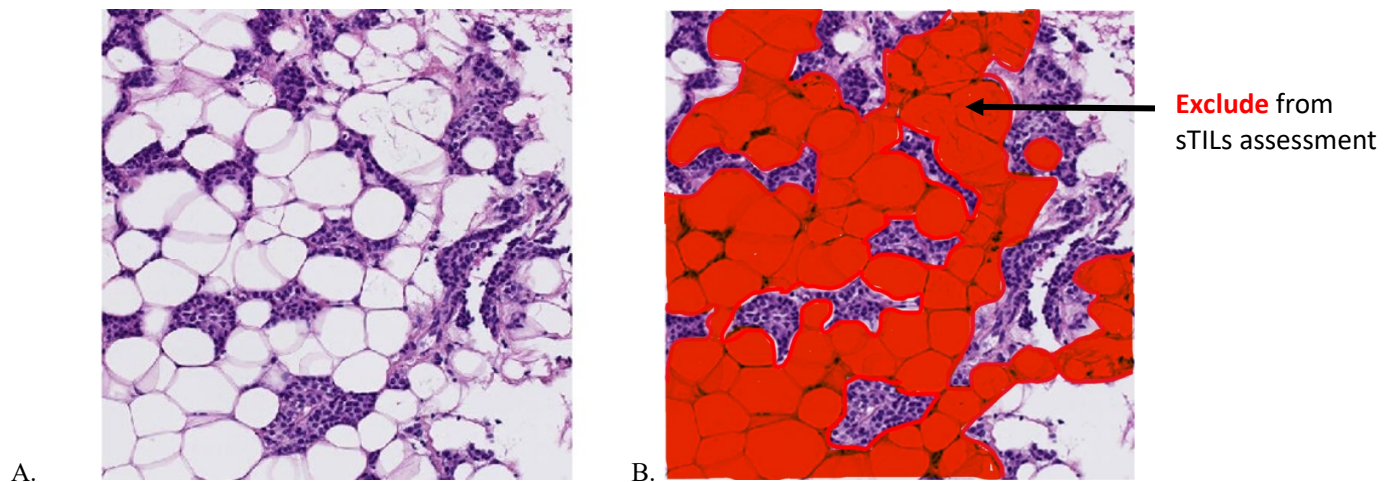


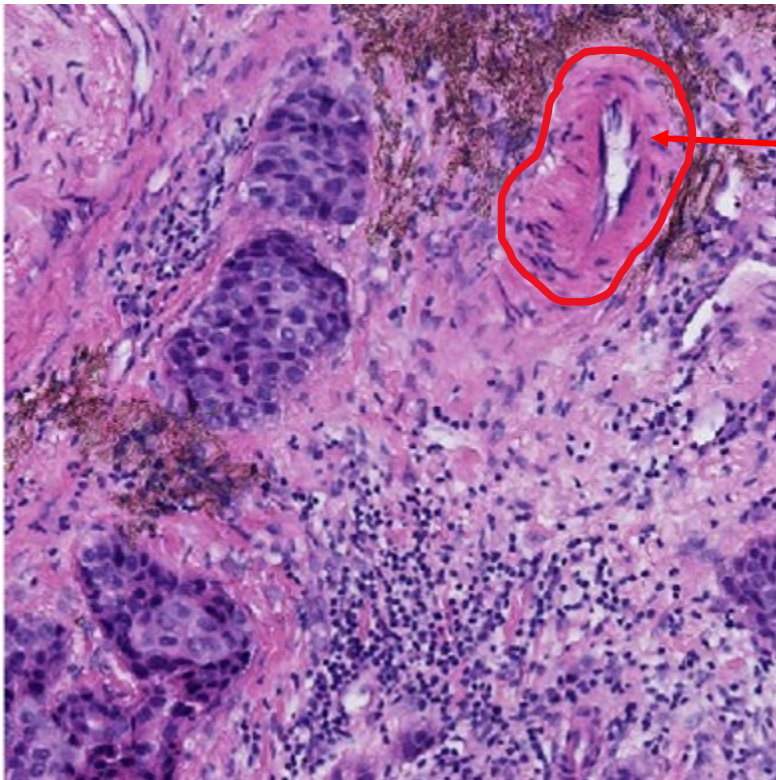
Figure 1: HTT-TILS-001-88B.ndpi_x38756.2190_y32439.2190

Mean Percent Tumor-Associated Stroma: 11.3%

Mean sTILs Density: 0.3%

Nerves and larger-caliber blood vessels

The walls of thick-walled vessels are not included as part of tumor-associated stroma, even if it is close to tumor cells and lymphocytes.



Exclude from sTILs assessment

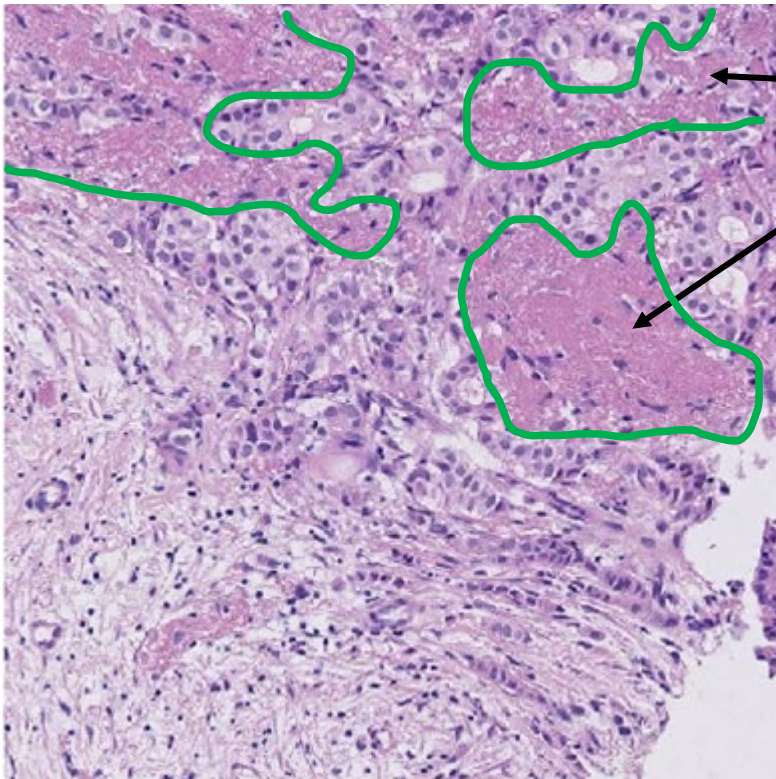
Figure 2: HTT-TILS-001-86B.ndpi_x20669.2190_y12026.2190

Mean Percent Tumor-Associated Stroma: 68.5%

Mean sTILs Density: 30%

Areas of necrosis and fibrin

Necrosis is excluded from the sTILs evaluation. Hyalinized and elastotic stroma are included in the assessment if associated with invasive carcinoma. Additionally, areas of elastosis and fibroblastic proliferation are included as tumor-associated stroma.



Hyalinized and elastotic stroma: **Include** in the sTILs assessment.

Correct discernment of necrosis versus elastotic or hyalinized stroma is important as necrosis is excluded from the sTILs assessment.

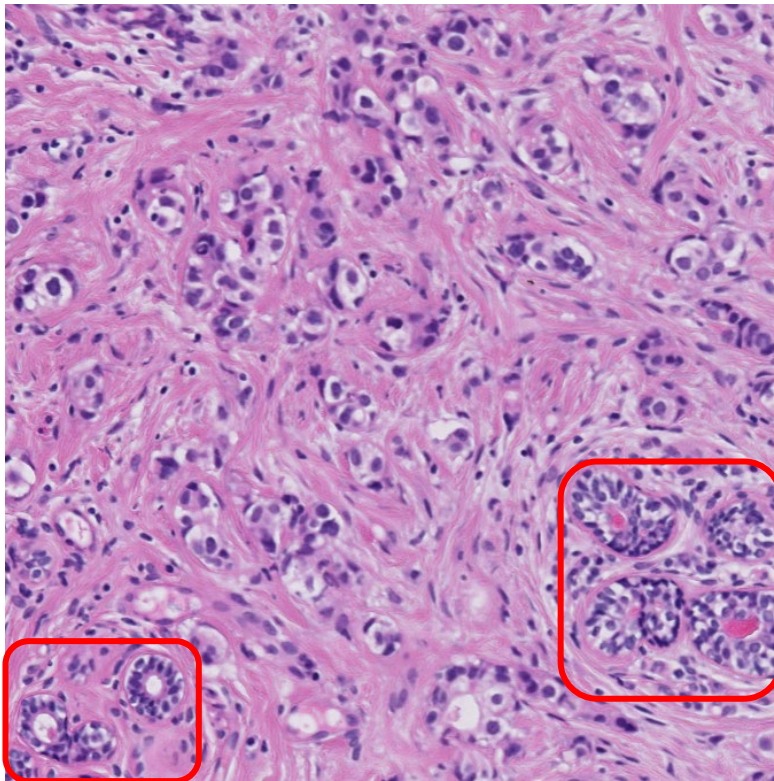
Figure 3: HTT-TILS-001-43B.ndpi_x12601.2190_y19050.2190

Mean Percent Tumor-Associated Stroma: 55%

Mean sTILs Density: 10.8%

Benign glandular elements, including intact terminal duct lobular units

Carcinoma in situ and benign glandular elements entrapped within the tumor area, including intact terminal duct lobular units, should be excluded from the numerator when calculating the percent of tumor-associated stroma. Stromal TILs surrounding carcinoma in situ should not be considered as sTILs, as sTILs are only associated with invasive cancer cells.



Benign terminal duct lobular unit: **Exclude** from sTILs assessment

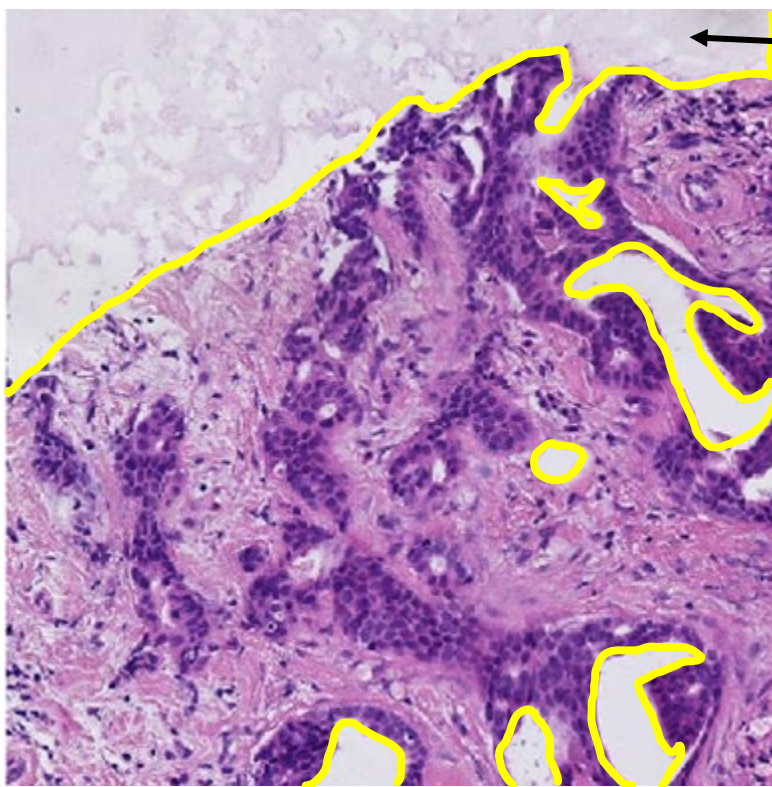
Figure 4: HTT-TILS-001-80B.ndpi_x56330.2190_y11332.2190

Mean Percent Tumor-Associated Stroma: 65.5%

Mean sTILs Density: 4.8%

2. The Percent of Tumor-Associated Stroma (Equation 1) is calculated with respect to the area of the entire ROI. Negative/empty space is included in the total ROI area, the denominator of the Percent Tumor-Associated Stroma, while the numerator is only the area of tumor-associated stroma.

While estimation of the percent of tumor-associated stroma is not performed in the clinical setting, for this validation dataset, we are assessing participants' ability to correctly identify tumor-associated stroma in a standardized approach. Vessel lumens, adipose tissue, negative/empty space, normal tissue, tumor cells, etc. are included in the entire ROI area. The below sample ROI contains negative/empty space in the upper left-hand corner, as well as empty spaces within several larger duct lumens. These empty spaces are included in the "area of entire ROI" when assessing the percent of tumor-associated stroma:



These yellow outlined areas are included in the "area of entire ROI" when assessing the percent of tumor-associated stroma.

They are excluded from the "area of tumor-associated stroma."

Figure 5: HTT-TILS-001-89B.ndpi_x16220.2190_y7255.2190

Mean Percent Tumor-Associated Stroma: 48.2%

Mean sTILs Density: 4.8%

Clinical Pearl: How far from tumor cells do we think of as tumor-associated stroma? Not a clear answer, even 1mm could be too much because could include normal tissue and their corresponding lymphocytes, which shouldn't be included.

3. Variations in cell morphology can make it difficult to distinguish stroma from tumor border.

Occasionally, tumor cells may exhibit cytoplasm with eosinophilia similar to that of adjacent stroma, and thus be mistaken for stroma, potentially affecting the sTILs score. Additional stains may be useful in these scenarios. A machine learning tool, which has been validated using the conventional invasive ductal phenotypes, may encounter difficulty when segmenting apocrine cancer cells showing eosinophilia similar to the adjacent stroma.

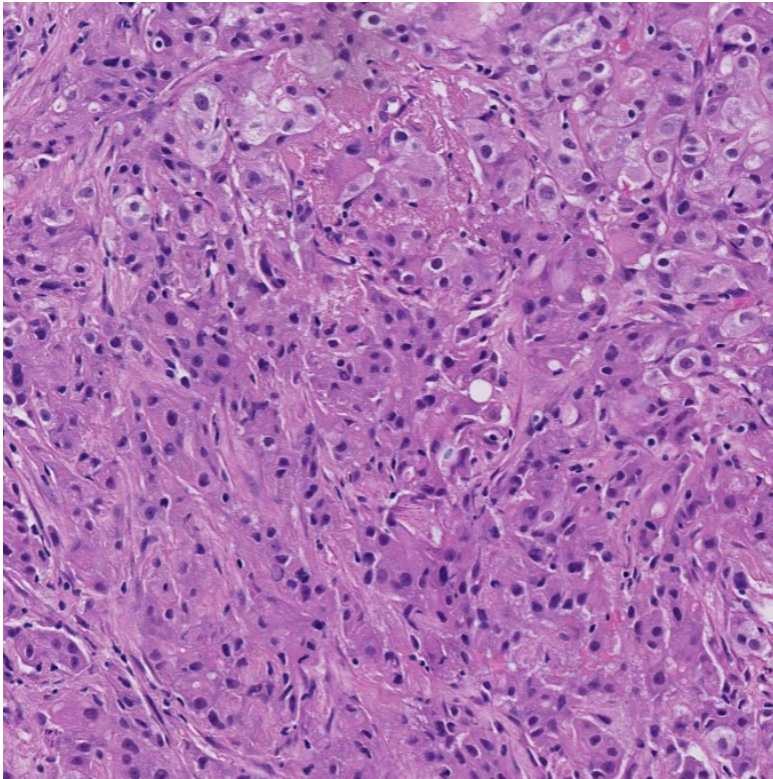


Figure 6: HTT-TILS-001-50B.ndpi_x39884.2190_y27444.2190

Mean Percent Tumor-Associated Stroma: 15%

Mean sTILs Density: 3.2%

sTILs Density Score

1. Cells with small/pyknotic nuclei and/or perinuclear clearing can be difficult to accurately categorize; such cells may be classified as macrophages, tumor cells, plasma cells, or lymphocytes. This may occur with invasive lobular carcinoma in particular, or in cases of suboptimal tissue fixation. Additional stains may be helpful.

TILs are limited to lymphocytes and plasma cells. Granulocytes, dendritic cells, lymphoid aggregates (tertiary lymphoid structures), and macrophages are not considered in the quantitative assessment. An example of cells with small/pyknotic nuclei and/or perinuclear clearing appear throughout the ROI in Figure 7.

In some of the examples in Section VI “Annotations and Discussions,” this pitfall is summarized as either “Perinuclear clearing can cause challenges in discrimination of cells, such as macrophages, lymphocytes/plasma cells, tumor cells, or others. Additional immunohistochemical staining may be helpful to further subclassify such cells” or “Non-lymphocytes with small nuclei may be confused for lymphocytes.”

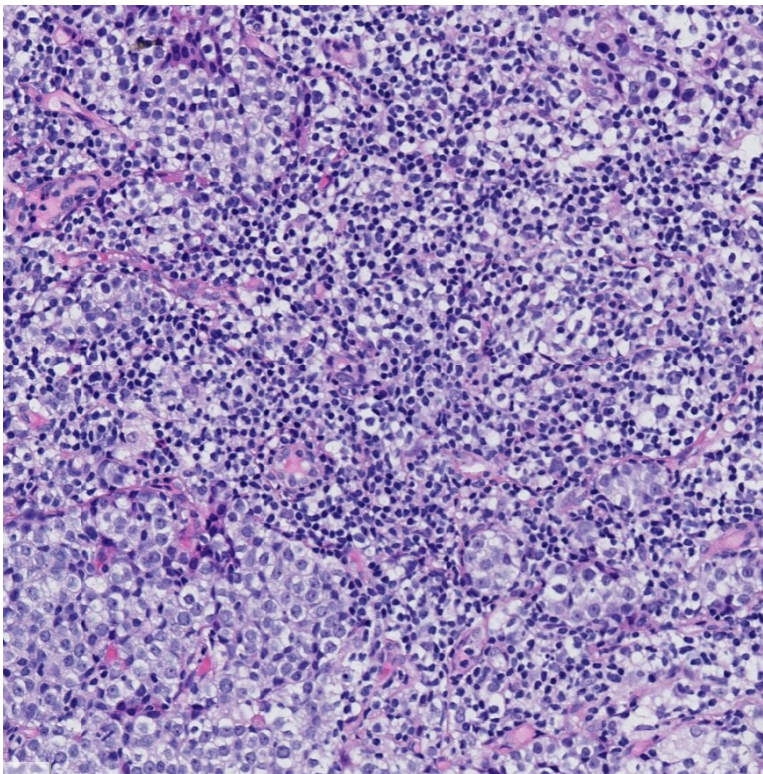


Figure 7: HTT-TILS-001-04B.ndpi_x24343.2190_y11775.2190
Mean Percent Tumor-Associated Stroma: 51.7%
Mean sTILs Density: 88.7%

2. The following non-lymphocytes may be confused for lymphocytes:

Cross-sectionally cut fibroblasts

Axially sectioned fibroblasts may be mistaken for lymphocytes, especially if the lymphocyte nuclei are the same size as fibroblasts. Without additional immunohistochemical staining, it would be hard to tell which cells are fibroblasts or lymphocytes. In Figure 8, fibroblasts can be seen throughout the ROI, excluding the two benign duct lobules in the 4 o'clock and 7 o'clock regions.

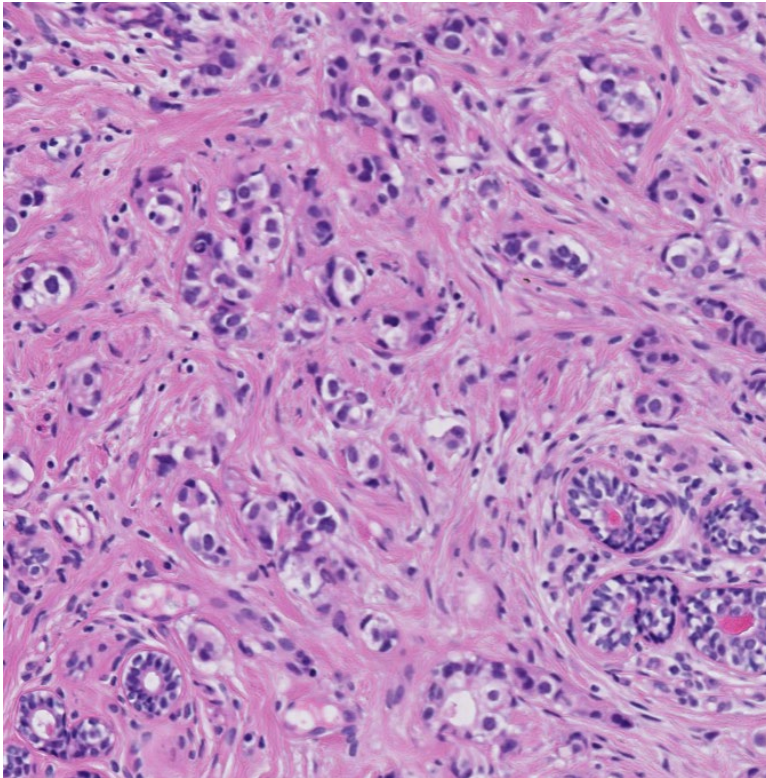


Figure 8: HTT-TILS-001-80B.ndpi_x56330.2190_y11332.2190

Mean Percent Tumor-Associated Stroma: 65.5%

Mean sTILs Density: 4.8%

Low grade and/or degenerated/ischemic tumor cells

Degenerating non-lymphocytes (e.g. pyknotic tumor cells) may be mistaken for lymphocytes. With low-grade atypia or apoptotic nuclei, one may confuse cancer cells as lymphocyte nuclei because they are very small and dark. In addition, sclerosing adenosis can mimic low-grade, infiltrating carcinoma. In Figure 9, these degenerate cells are centered around the pink branched stromal structures extending between the 9-12 o'clock regions and in the bottom right-hand corner.

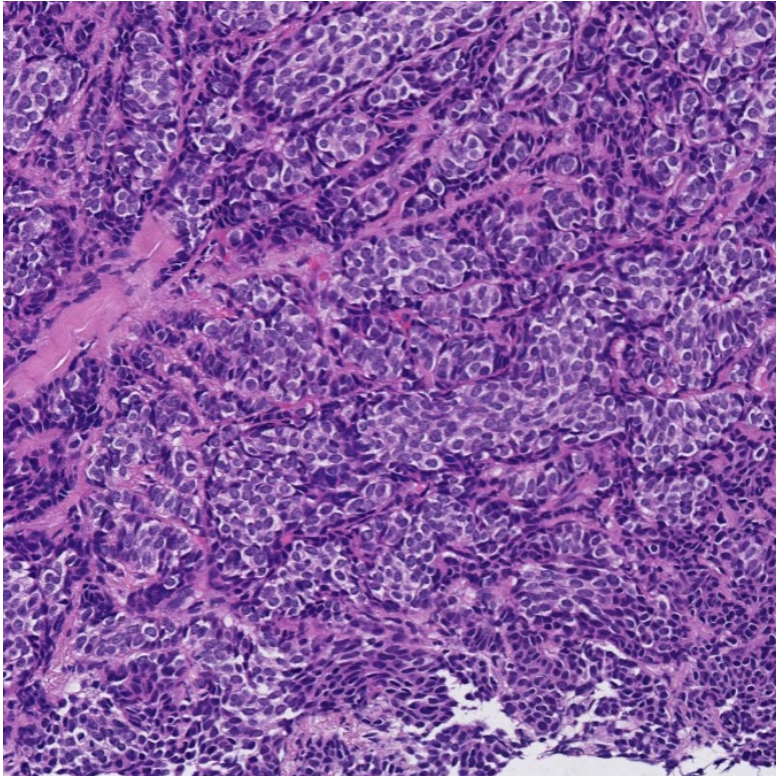


Figure 9: HTT-TILS-001-52B.ndpi_x71829.2190_y49452.2190

Mean Percent Tumor-Associated Stroma: 14.3%

Mean sTILs Density: 1%

Crushed cells of any type

Non-lymphocytes may be confused for lymphocytes if there are tissue fixation and/or cellular preservation artifacts. Crush artifacts hinder our ability to be certain about our sTILs assessment. An example of crushed cells is shown in the below ROI by the region outline in yellow.

In some of the examples in Section VI “Annotations and Discussions,” additional artifact-related pitfalls include: “There will be areas out-of-focus because there are different thicknesses across the whole region, or bad scan quality can contribute to an inaccurate assessment of sTILs” and “Ischemic artefacts illustrate that the pre-analytical conditions are crucial for a correct interpretation of TILs, either for manual assessment or using Machine Learning-tools.”

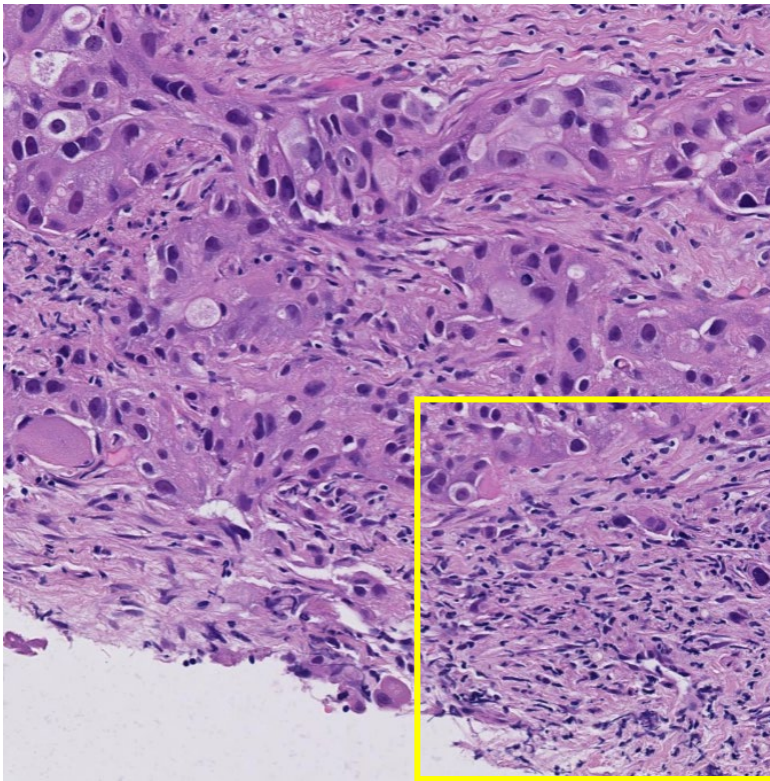


Figure 10: HTT-TILS-001-50B.ndpi_x20589.2190_y28549.2190

Mean Percent Tumor-Associated Stroma: 37.7%

Mean sTILs Density: 26%

Clinical Pearls: One may try to mentally compress the stroma in one corner and visualize how much space it takes up of the entire ROI. If you put all the tumor in one quadrant, it will not fill one quadrant, so the tumor area is less than 25%.

3. Error in the percent tumor associated stroma can lead to inflated or deflated sTILs scores.

Stroma may be obscured by dense populations of cells and may be incorrectly excluded from the sTILs evaluation. This can result in the sTILs score being inflated with a lower estimated percent stroma. Dense populations of lymphocytes can be seen throughout Figures 7 and 11.

In some of the examples in Section VI “Annotations and Discussions,” this pitfall is summarized as either: “In regions where the sTILs density is very high, the underlying stroma may be obscured” or “Error in percent tumor-associated stroma can affect sTILs score.”

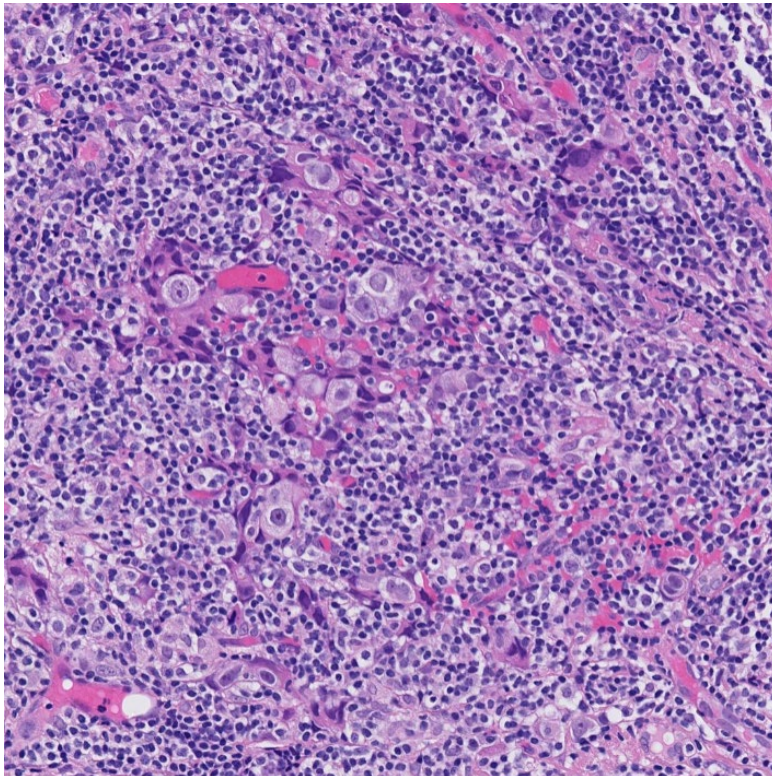


Figure 11: HTT-TILS-001-

76B.ndpi_x24500.2190_y76408.2190

Mean Percent Tumor-Associated Stroma: 76.7%

Mean sTILs Density: 92.5%

4. Human Cognitive Error

When tumor cells (or stroma) are sparsely distributed throughout the ROI, it may be more challenging to accurately quantitate the percent tumor-associated stroma and sTILs density (see Figure 11). In some of the examples in Section VI “Annotations and Discussions,” this pitfall is expressed as “The sTILs assessment may be more difficult when tumor cells are sparse and scattered throughout the ROI.”

V. Clinical Pearls for Approaching sTILs Assessment

Below are a series of quotes and summaries of the cognitive processes used by our experts when performing their sTILs assessment. Some of the quotes are accompanied by the image that inspired the quote. The focus of the quotes is on their approach to determining the sTILs density. We feel these discussions add depth to the training and help annotators with their assessment.

1. Pathologists may have a difficult time concluding whether the sTILs density is 1% or 6%, but there will generally be tight agreement that it is low. The key is consistency in your process. sTILs are currently reported as a continuous variable with no established prognostic categories or cut-offs; this is an area that needs more data/clinical impact clarification.

2. When I evaluate TILs, I draw the line of 50%, and consider above or less. I think this is more than 50%. Between 50-100%, it's probably somewhere in the middle, so 60-70% will be good. Because there is still a lot of empty stroma in the lower left-hand corner, I think 60%. I'm also influenced by the tumor cellularity guide on the MD Anderson Cancer website (<https://www.mdanderson.org/education-and-research/resources-for-professionals/clinical-tools-and-resources/clinical-calculators/calculators-cellularity-guide.pdf>). Our eyes tend to overestimate the cellularity, so I tend to be very conservative of evaluating the tumor cellularity. (See Figure 12)

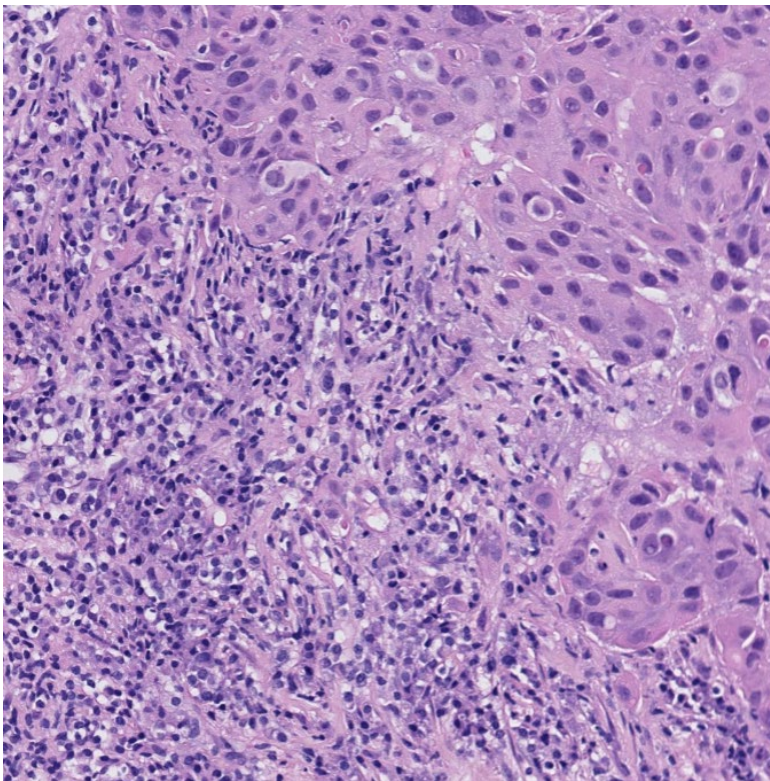


Figure 12: HTT-TILS-001-33B.ndpi_x29652.2190_y6224.2190
Mean Percent Tumor-Associated Stroma: 61.7%

Mean sTILs Density: 75.8%

3. "I can never reach 100% sTILs density because there is always some kind of space between the cells."
(See Figure 13)

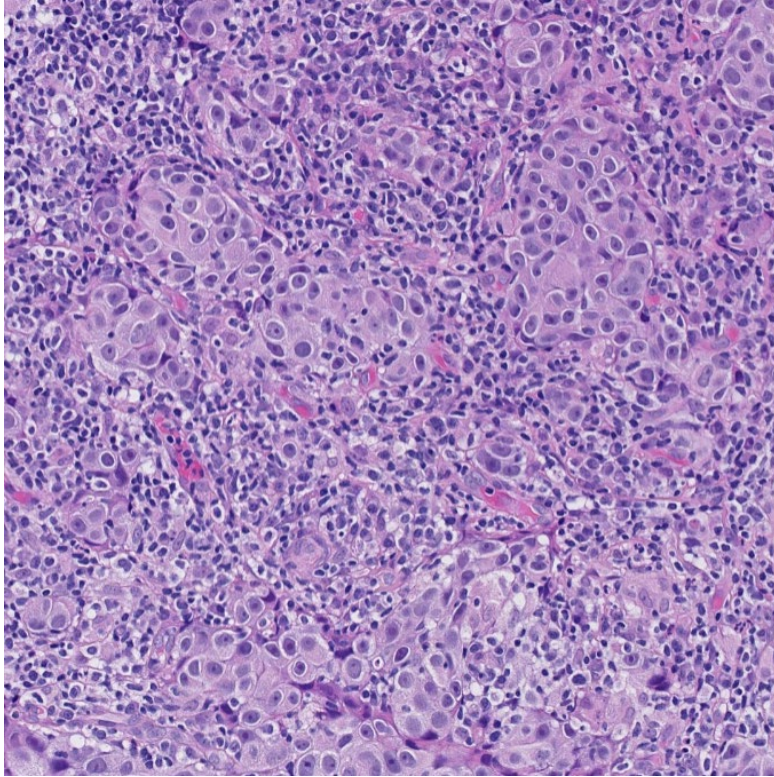


Figure 13: HTT-TILS-001-76B.ndpi_x15338.2190_y44740.2190

Mean Percent Tumor-Associated Stroma: 43.7%

Mean sTILs Density: 93.7%

4. We do not need to evaluate tumor cellularity, only tumor area proportion. Some AI/ML tools perform according to cell counts and not according to area segmentation. A score based on cell count would be a different result than those based on tumor area. (See Figure 14)

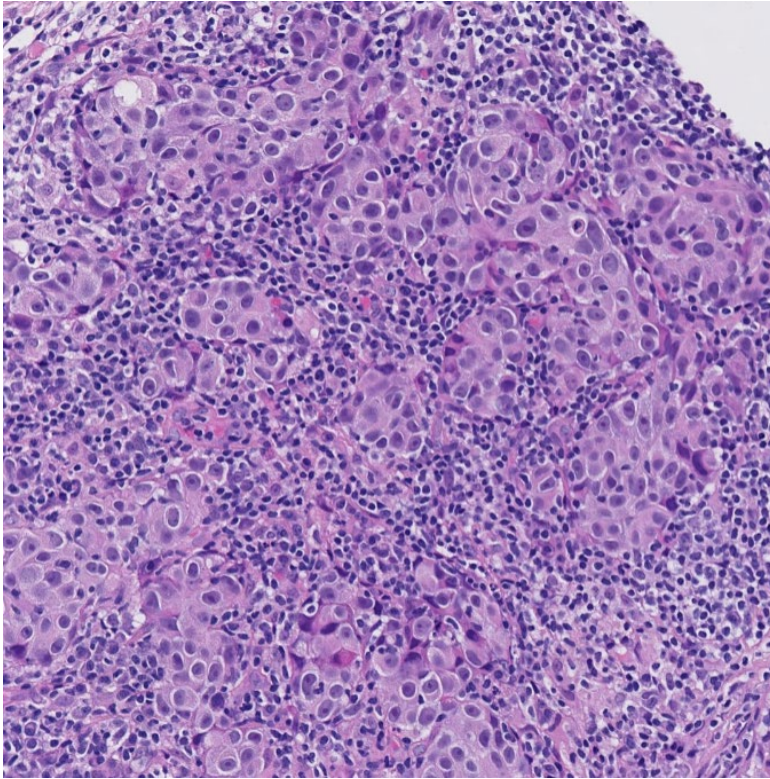


Figure 14: HTT-TILS-001-76B.ndpi_x16170.2190_y42769.2190

Mean Percent Tumor-Associated Stroma: 35.8%

Mean sTILs Density: 91.8%

5. The most concentrated area of TILs in this ROI is the middle of the lower portion. For this area, there are 40 to 50% TILs. However, this area is about one ninth of the entire ROI. There are other areas with some lymphocytes, but it is very minimal. So, if I divide 50% by nine that will give me less than 10%. I can be more accurate, but I would say, 7-8% of the entire tumor-associated stromal area is TILs. (See Figure 15)

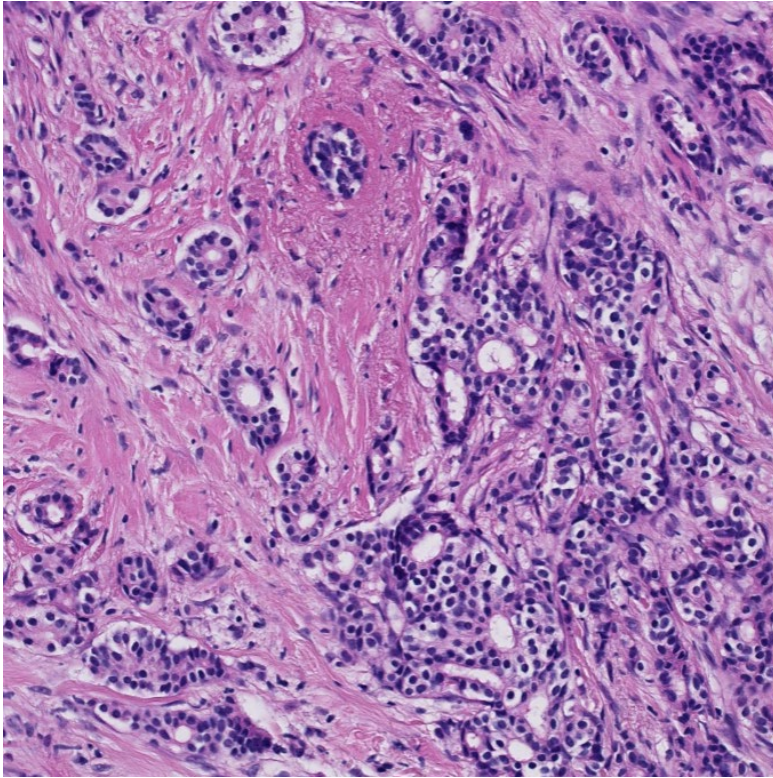


Figure 15: HTT-TILS-001-32B.ndpi_x34191.2190_y65798.2190

Mean Percent Tumor-Associated Stroma: 48.2%
Mean sTILs Density: 5%

6. An expert's thought process on determining if a cell is a lymphocyte: The interesting thing in that case is that the TILs didn't look like the classical textbook TILs. TILs are lymphocytes and plasma cells, and these cells have a type of perinuclear clearing. So, they looked a bit different and may be macrophages. But, they are probably TILs with artifact and are conceptually considered as TILs. If you use a machine learning tool that uses a lymph node for its training example, it may not be calibrated to identify these cells. (See Figure 16)

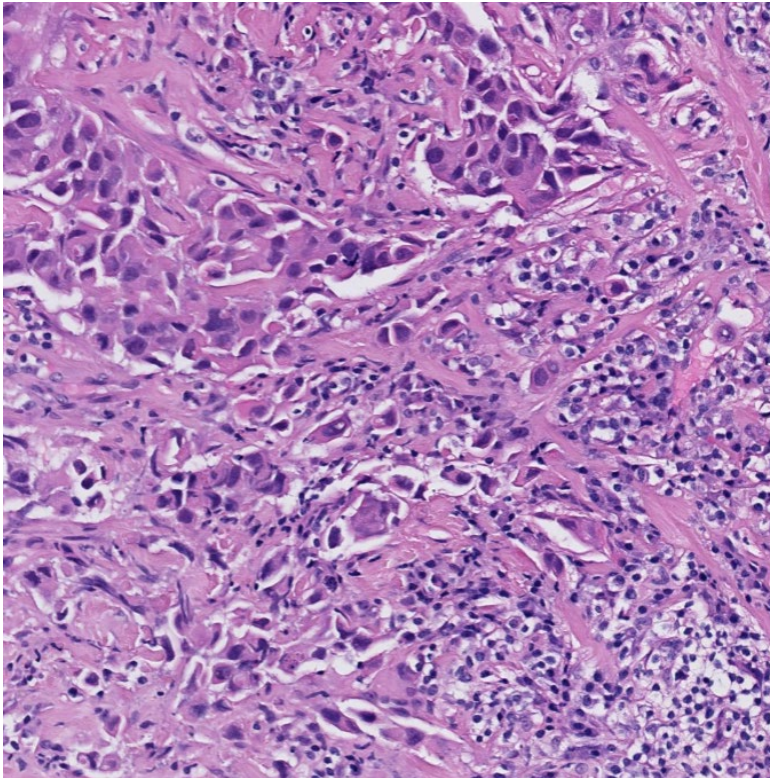
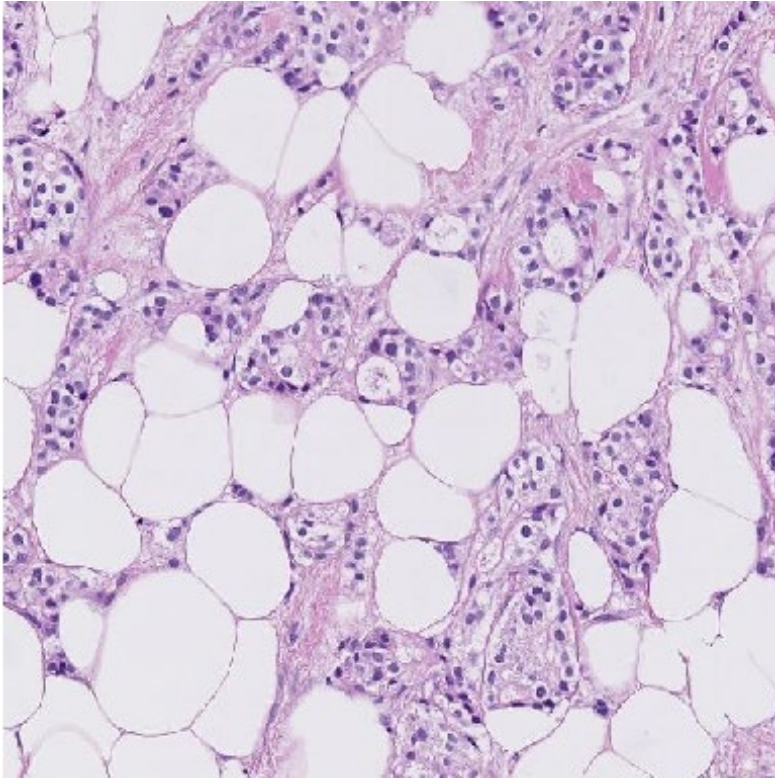


Figure 16: HTT-TILS-001-33B.ndpi_x29809.2190_y47938.2190

Mean Percent Tumor-Associated Stroma: 60%

Mean sTILs Density: 43.7%

VI. Annotations and Discussions



caseID: HTT-TILS-001-03B.ndpi_x4934.2190_y68036.2190

Expert Panel Annotations

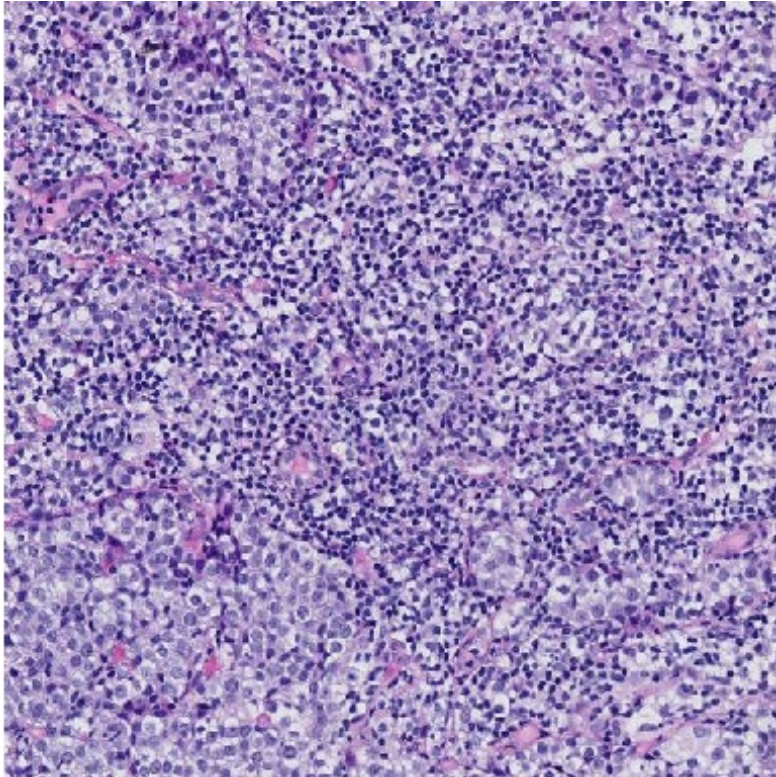
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	25	0
Evaluable	12	5
Evaluable	25	5
Evaluable	25	0
Evaluable	40	0
Evaluable	50	2

Mean Percent Tumor-Associated Stroma: 29.5

Mean sTILs Density: 2

Comments: Adipocytes are excluded from tumor-associated stroma.

Pitfalls: Adipocytes are not considered part of the tumor-associated stroma for purposes of sTILs assessment.



caseID: HTT-TILS-001-04B.ndpi_x24343.2190_y11775.2190

Expert Panel Annotations

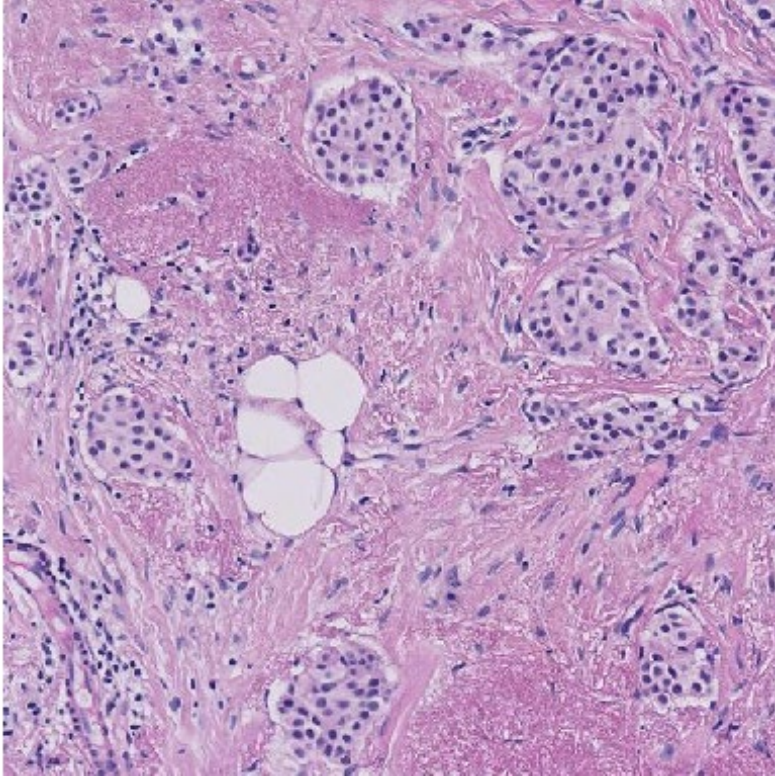
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	30	90
Evaluable	60	95
Evaluable	50	92
Evaluable	50	75
Evaluable	60	90
Evaluable	60	90

Mean Percent Tumor-Associated Stroma: 51.7

Mean sTILs Density: 88.7

Comments: A challenging case. The high density of lymphocytes results in difficulty determining whether the lymphocytes are located in stroma, or whether they infiltrate tumor cell nests. The presence of small blood vessels and small gaps between lymphocytes suggest the lymphocytes reside within stroma. Occasional tumor cells with small nuclei (possibly degenerating) may be confused for lymphocytes.

Pitfalls: In regions where the sTILs density is very high, the underlying stroma may be obscured. Non-lymphocytes with small nuclei may be confused for lymphocytes.



caseID: HTT-TILS-001-27B.ndpi_x29456.2190_y23838.2190

Expert Panel Annotations

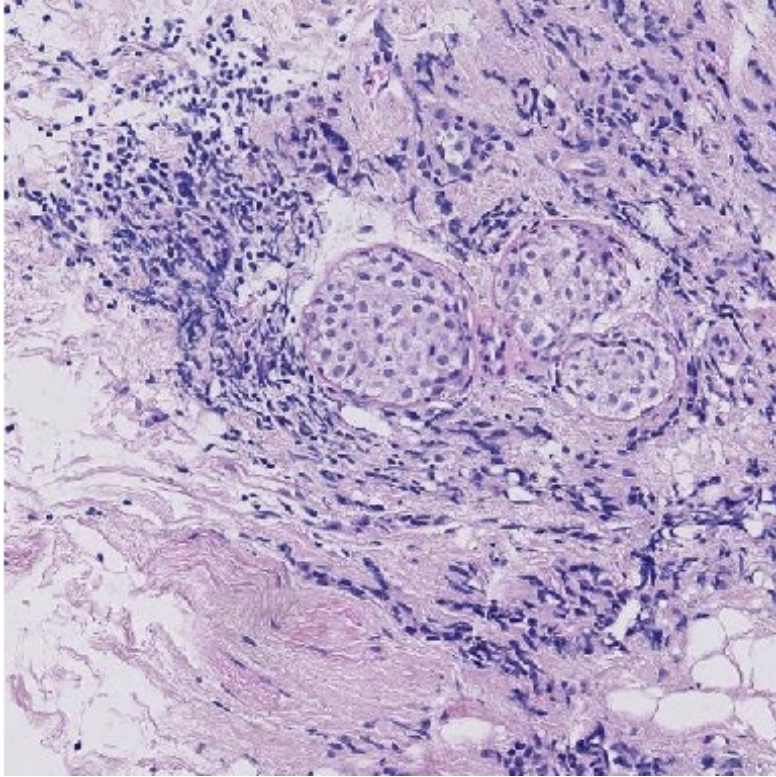
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	70	3
Evaluable	60	8
Evaluable	75	8
Evaluable	70	3
Evaluable	75	1
Evaluable	60	5

Mean Percent Tumor-Associated Stroma: 68.3

Mean sTILs Density: 4.7

Comments: There is clearly demarcated stroma in invasive tumor.

Pitfalls: Adipocytes are not considered part of the tumor-associated stroma for purposes of sTILs assessment. Areas of elastosis and fibroblastic proliferation are included as tumor-associated stroma.



caseID: HTT-TILS-001-27B.ndpi_x5114.2190_y25709.2190

Expert Panel Annotations

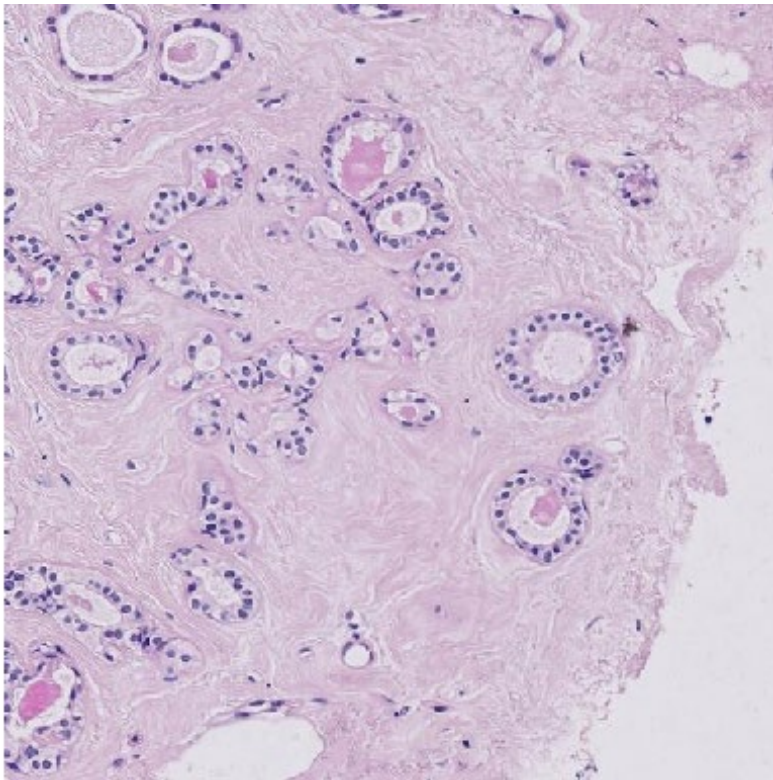
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	60	15
Evaluable	8	20
Evaluable	75	13
Evaluable	70	3
Evaluable	80	10
Evaluable	80	10

Mean Percent Tumor-Associated Stroma: 62.2

Mean sTILs Density: 11.8

Comments: A challenging ROI. The DCIS in the center of the ROI is excluded from the calculation. Crushed tumor cells may be confused for lymphocytes. There are few small foci of invasive tumor (e.g. in upper third of ROI) that would be the focus of assessment. The lower part of ROI is also not reliable for scoring due to marked crush artifact. Crushed cells in the lower right quarter are suspicious for carcinoma, and not TILs, based on architecture similar to tumor cells outside of this ROI.

Pitfalls: DCIS is excluded from the numerator when calculating the percentage of tumor-associated stroma. Non-lymphocytes may be confused for lymphocytes if there is tissue fixation/preservation artifact.



caseID: HTT-TILS-001-29B.ndpi_x12694.2190_y13922.2190

Expert Panel Annotations

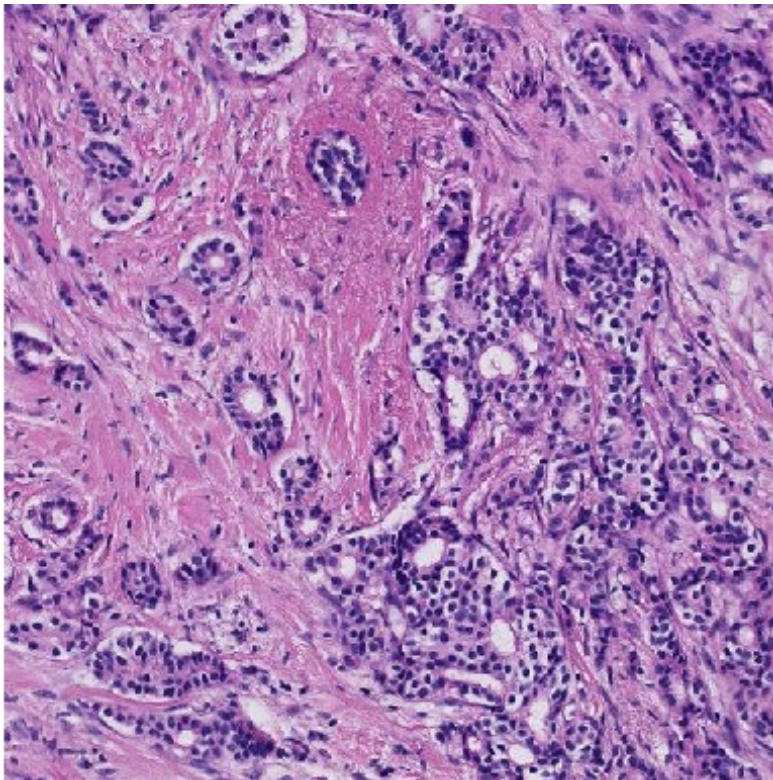
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Not Evaluable	NA	NA

Mean Percent Tumor-Associated Stroma: NA

Mean sTILs Density: NA

Comments: A difficult case that would need further staining to confidently determine if could be tumor.

Pitfalls: Invasive carcinoma must be present to perform the sTILs assessment.



caseID: HTT-TILS-001-32B.ndpi_x34191.2190_y65798.2190

Expert Panel Annotations

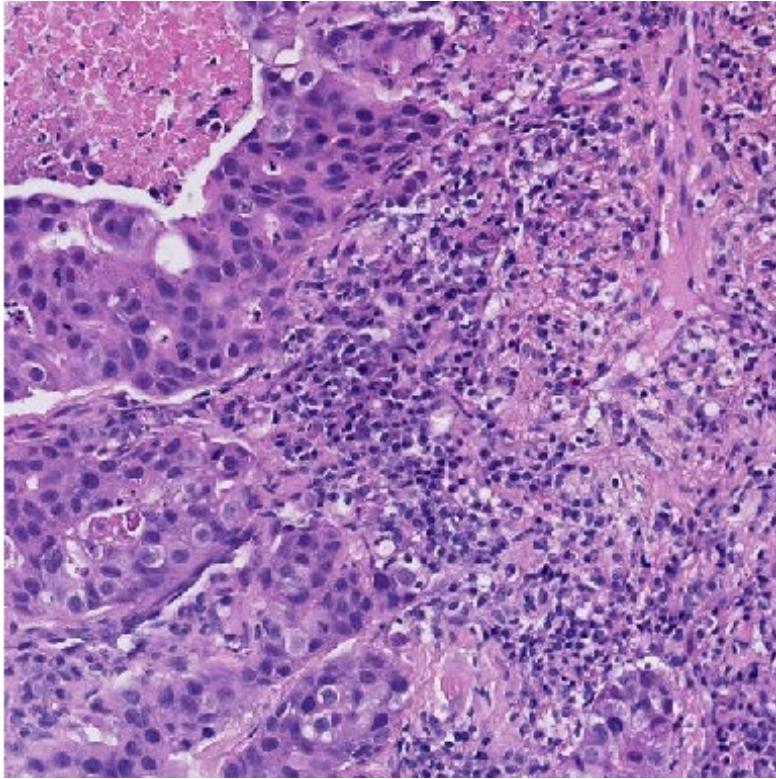
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	50	5
Not Evaluable	NA	NA
Evaluable	31	10
Evaluable	65	5
Evaluable	45	0
Evaluable	50	5

Mean Percent Tumor-Associated Stroma: 48.2

Mean sTILs Density: 5

Comments: A subset of carcinoma cells exhibit perinuclear clearing/halos (e.g. 5 o'clock). Because these cells form cohesive nests and tubules, they can be recognized as carcinoma, and not lymphocytes. In addition, when assessing for percent tumor-associated stroma, exclude gland lumen from the area of tumor-associated stroma.

Pitfalls: Perinuclear clearing can cause challenges in discrimination of cells, such as macrophages, lymphocytes/plasma cells, tumor cells, or others. Additional immunohistochemical staining may be helpful to further subclassify such cells.



caseID: [HTT-TILS-001-33B.ndpi_x25888.2190_y33488.2190](#)

Expert Panel Annotations

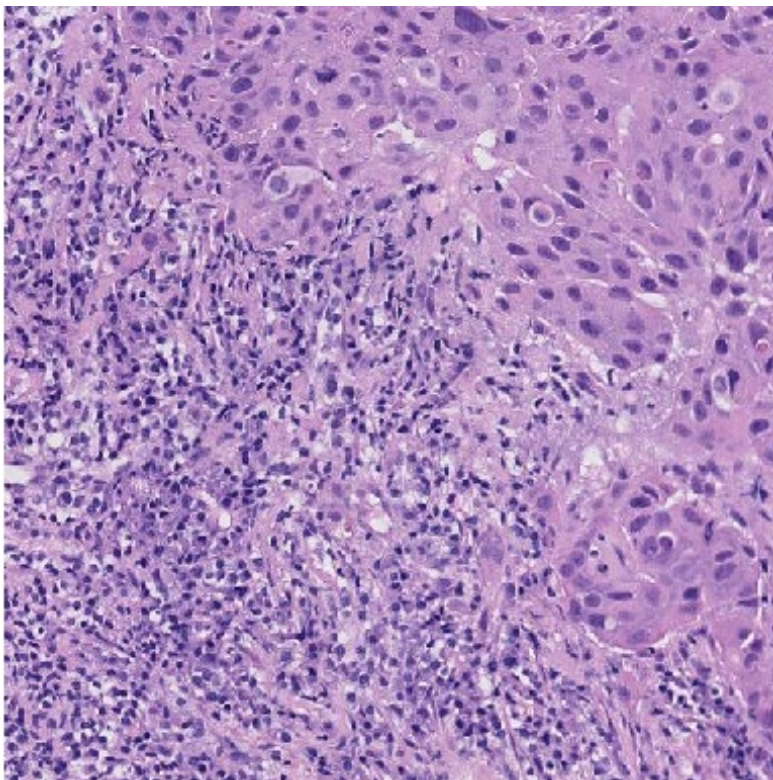
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	60	50
Evaluable	55	75
Evaluable	56	70
Evaluable	46	75
Evaluable	60	75
Evaluable	50	40

Mean Percent Tumor-Associated Stroma: 54.5

Mean sTILs Density: 64.2

Comments: Around 2 o'clock, cells with perinuclear halos and eosinophilic cytoplasm could be plasma cells or macrophages. This ambiguity is due to the slide fixation, and they are likely all the same cell type; immunohistochemical staining could confirm their cell type(s). There was a considerable range of sTILs density scores among our experts; the 2 lower density scores of 40 and 50 may be a result of the perinuclear clearing in the 3 o'clock region centrally causing uncertainty as to whether those cells are macrophages or lymphocytes. There are also fibroblasts present that may be mistaken for TILs.

Pitfalls: Perinuclear clearing can cause challenges in discrimination of cells, such as macrophages, lymphocytes/plasma cells, tumor cells, or others. Additional immunohistochemical staining may be helpful to further subclassify such cells.



caseID: HTT-TILS-001-33B.ndpi_x29652.2190_y6224.2190

Expert Panel Annotations

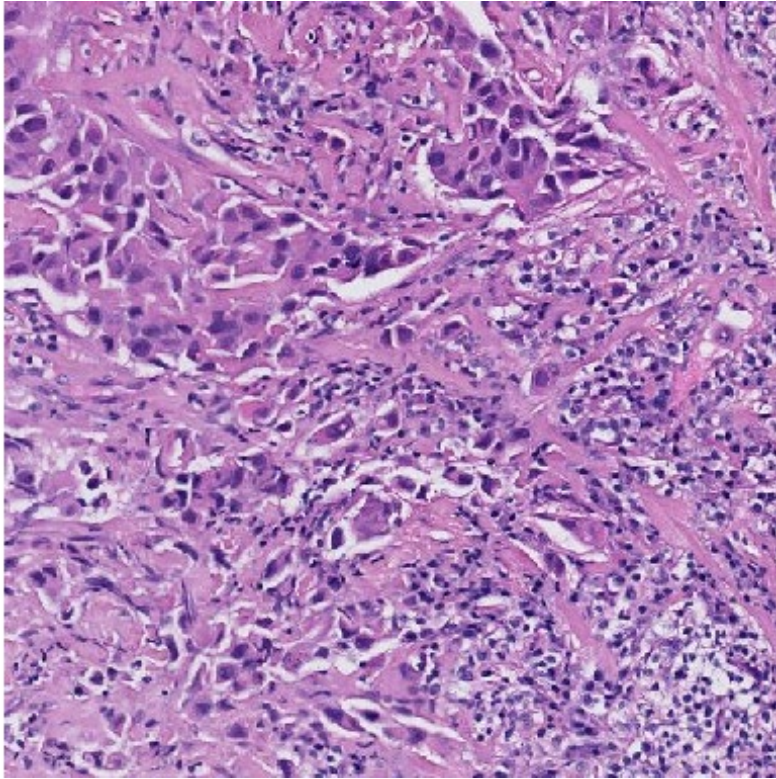
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	70	85
Evaluable	60	70
Evaluable	50	75
Evaluable	70	85
Evaluable	60	80
Evaluable	60	60

Mean Percent Tumor-Associated Stroma: 61.7

Mean sTILs Density: 75.8

Comments: “This one’s easy in a sense, because the square is very nicely divided in triangles somewhat ... it’s more than 50% of stroma and so it’s 55-60% stroma....” Despite perinuclear halos being prominent in this ROI, experts achieved better concordance; this is likely due to the arrangement of tumor and stroma in the ROI, where the tumor is all aggregated to one aspect.

Pitfalls: There are no significant pitfalls for this ROI.



caseID: [HTT-TILS-001-33B.ndpi_x29809.2190_y47938.2190](#)

Expert Panel Annotations

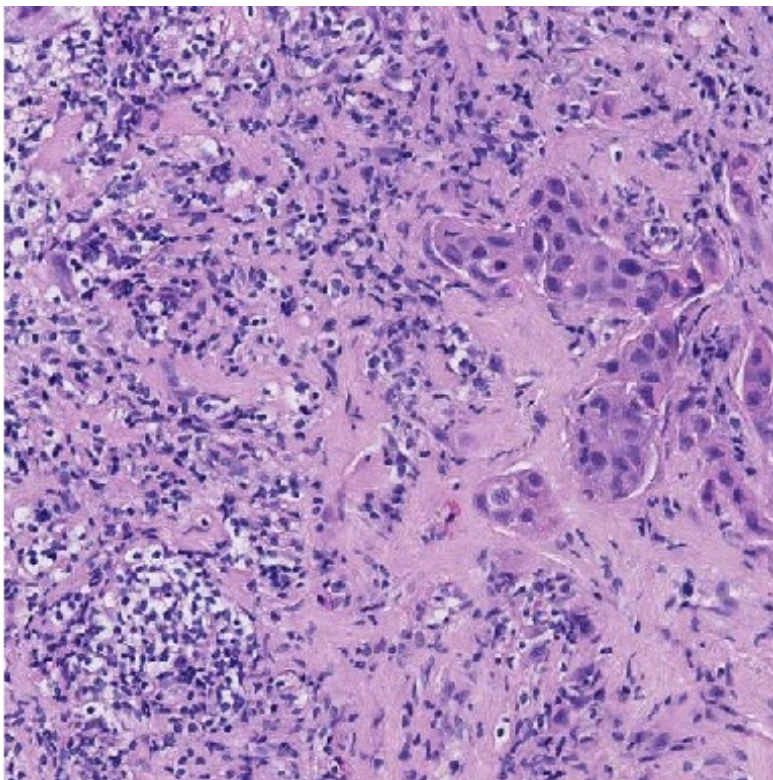
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	60	30
Evaluable	60	30
Evaluable	50	67
Evaluable	75	50
Evaluable	65	45
Evaluable	50	40

Mean Percent Tumor-Associated Stroma: 60

Mean sTILs Density: 43.7

Comments: This is a suboptimal H&E fixation with lots of cellular degeneration. It has been demonstrated that poor staining and fixation has been associated with greater discordance among pathologist scores (Kos et al., *npj Breast Cancer* (2020), doi.org/10.1038/s41523-020-0156-0). This can lead to difficulty determining cell types, where immunohistochemical staining could confirm their cell type(s). Due to the problematic nature of this ROI, it may not be an ideal region to use in clinical practice.

Pitfalls: Perinuclear clearing can cause challenges in discrimination of cells, such as macrophages, lymphocytes/plasma cells, tumor cells, or others. Additional immunohistochemical staining may be helpful to further subclassify such cells.



caseID: HTT-TILS-001-33B.ndpi_x32624.2190_y18730.2190

Expert Panel Annotations

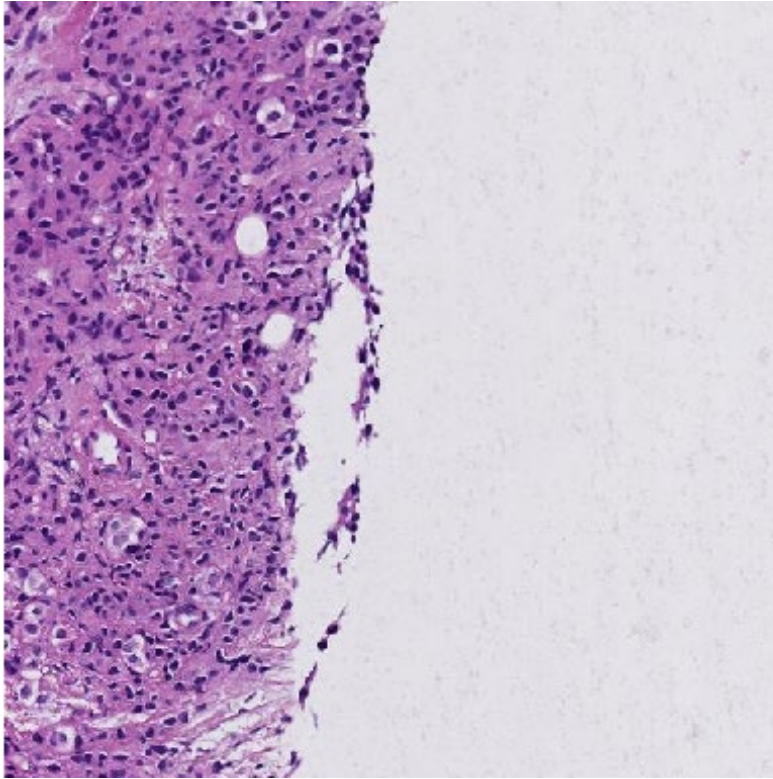
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	85	40
Evaluable	80	35
Evaluable	86	61
Evaluable	85	65
Evaluable	80	50
Evaluable	85	30

Mean Percent Tumor-Associated Stroma: 83.5

Mean sTILs Density: 46.8

Comments: The perinuclear clearing in the 6-9 o'clock region can cause difficulty distinguishing between macrophages and lymphocytes. Crush artifact (at 9 and 12 o'clock) may challenge the ability to categorize cells. Additional immunohistochemical staining could confirm their cell types(s). The uneven distribution of cell, especially the density differences between the right and left halves of the ROI, introduces additional uncertainty in determining an sTILs density for the entire ROI.

Pitfalls: Non-lymphocytes may be confused for lymphocytes if there is cellular preservation artifact.



caseID: HTT-TILS-001-34B.ndpi_x13400.2190_y32583.2190

Expert Panel Annotations

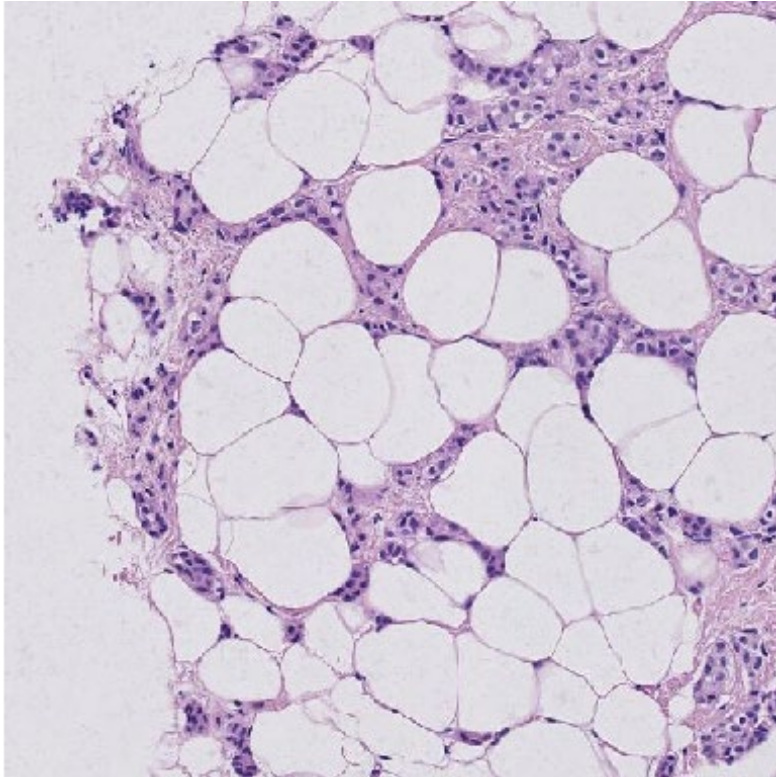
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	5	0
Evaluable	5	5
Evaluable	8	5
Evaluable	15	3
Evaluable	10	0
Evaluable	15	10

Mean Percent Tumor-Associated Stroma: 9.7

Mean sTILs Density: 3.8

Comments: Tumor cell eosinophilia is similar in appearance to stromal eosinophilia and causes difficulty in distinguishing these entities. The sTILs density can be inflated due to the edge artifact (upper part of lower zone) and crushed zone. An algorithm may recognize these as intraepithelial TILs since they are smaller than the cells in the upper part of the ROI.

Pitfalls: Occasionally, tumor cells may exhibit cytoplasm with eosinophilia similar to that of adjacent stroma, and thus be mistaken for stroma, potentially affecting the sTILs score. Non-lymphocytes may be confused for lymphocytes if there is tissue fixation artifact. Non-lymphocytes with small nuclei may be confused for lymphocytes.



caseID: [HTT-TILS-001-41B.ndpi_x2572.2190_y8345.2190](#)

Expert Panel Annotations

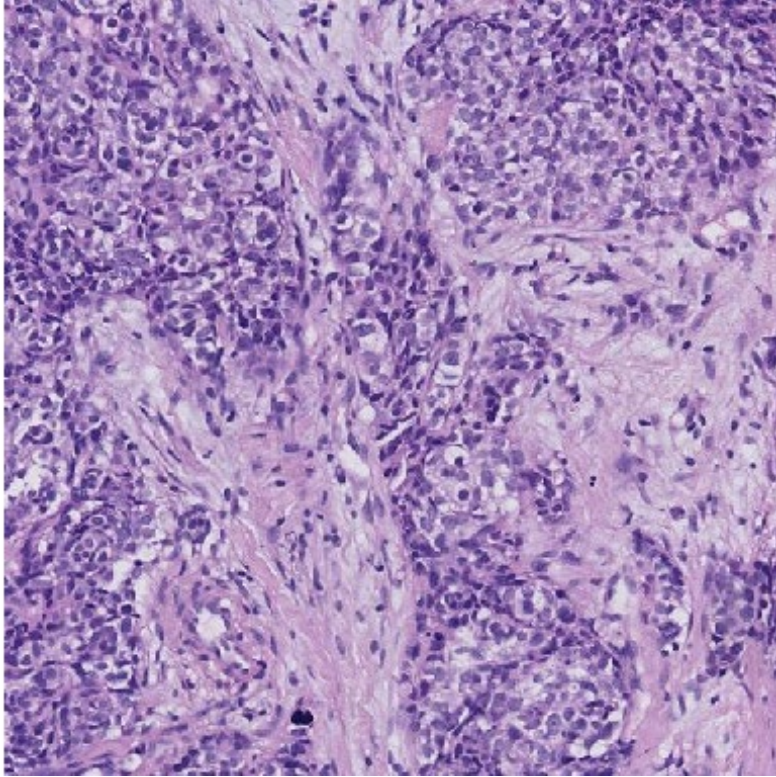
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	10	0
Evaluable	5	1
Evaluable	14	4
Evaluable	20	0
Evaluable	40	0
Evaluable	50	2

Mean Percent Tumor-Associated Stroma: 23.2

Mean sTILs Density: 1.2

Comments: Two pitfalls in assessing Percent Tumor-Associated Stroma are captured in this ROI. Adipocytes are excluded from tumoral stroma. In addition, there is empty space that one must remember to include when calculating the stromal percentage. One may try to mentally compress the stroma in one corner and visualize how much space it takes up of the entire ROI.

Pitfalls: Adipocytes are not considered part of tumor-associated stroma for purposes of sTILs assessment. The percent of tumor-associated-stromal is calculated with respect to the area of the entire ROI. Negative/empty space is in the total ROI area, the denominator of the Percent Tumor-Associated Stroma.



caseID: HTT-TILS-001-42B.ndpi_x9321.2190_y18852.2190

Expert Panel Annotations

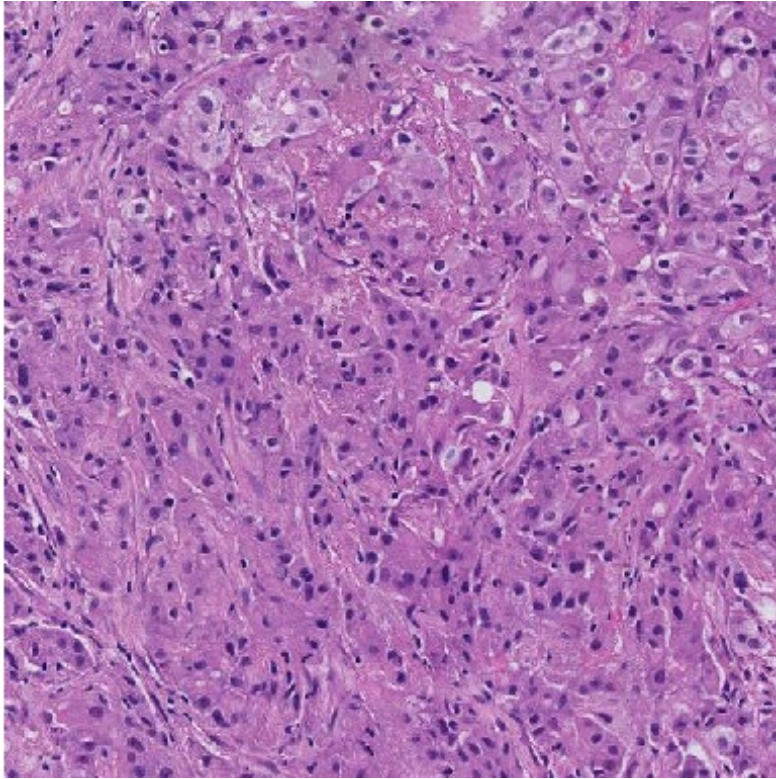
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	30	5
Evaluable	40	9
Evaluable	50	7
Evaluable	50	3
Evaluable	40	1
Evaluable	50	5

Mean Percent Tumor-Associated Stroma: 43.3

Mean sTILs Density: 5

Comments: It is difficult to distinguish between fibroblasts and sTILs in this case. The cells in the middle of the ROI are a bit wider than the other cells, so they probably are cancer cells that have artifact as a result of tissue processing. Though strong suspicion for a cancer cell, it could be a macrophage, which we see after treatment, and expect that an algorithm will have difficulty making this distinction on H&E stain.

Pitfalls: Non-lymphocytes may be confused for lymphocytes if there is tissue fixation artifact. Axially sectioned fibroblasts may be mistaken for lymphocytes.



caseID: HTT-TILS-001-50B.ndpi_x39884.2190_y27444.2190

Expert Panel Annotations

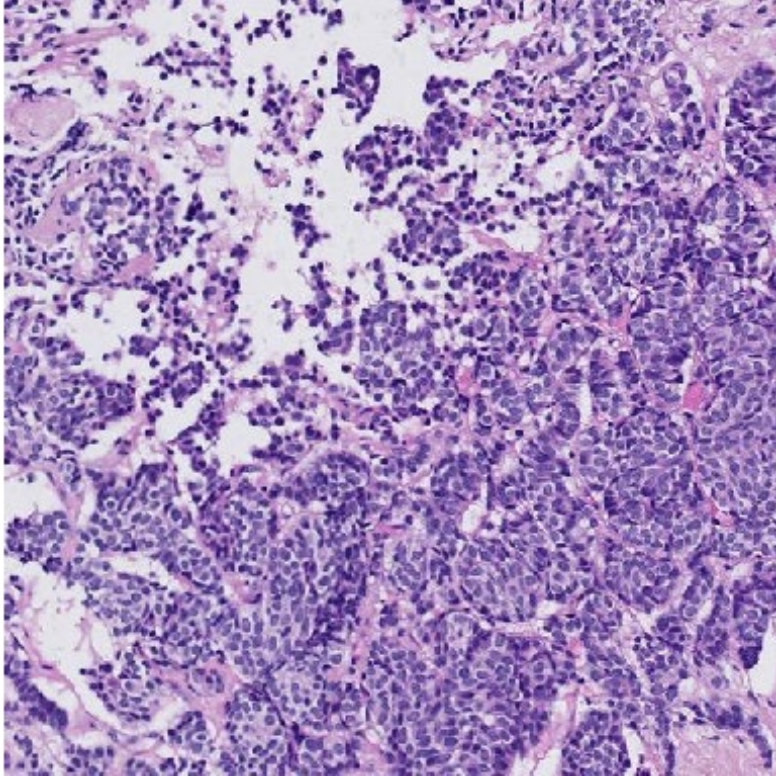
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Not Evaluable	NA	NA
Evaluable	15	5
Evaluable	15	4
Evaluable	10	5
Evaluable	20	1
Evaluable	15	1

Mean Percent Tumor-Associated Stroma: 15

Mean sTILs Density: 3.2

Comments: This intra-tumoral stroma case is a “good case because cells are very pink, as much as pink as the stroma in between. So, it’s not so easy to identify the stroma which is in between the cancer cells.” Looking between three and six o’clock, this region is harder to assess than those in the upper right because you can’t see the cell borders very clearly.

Pitfalls: Occasionally, tumor cells may exhibit cytoplasm with eosinophilia similar to that of adjacent stroma, and thus be mistaken for stroma, potentially affecting the sTILs score.



caseID: HTT-TILS-001-52B.ndpi_x22110.2190_y13641.2190

Expert Panel Annotations

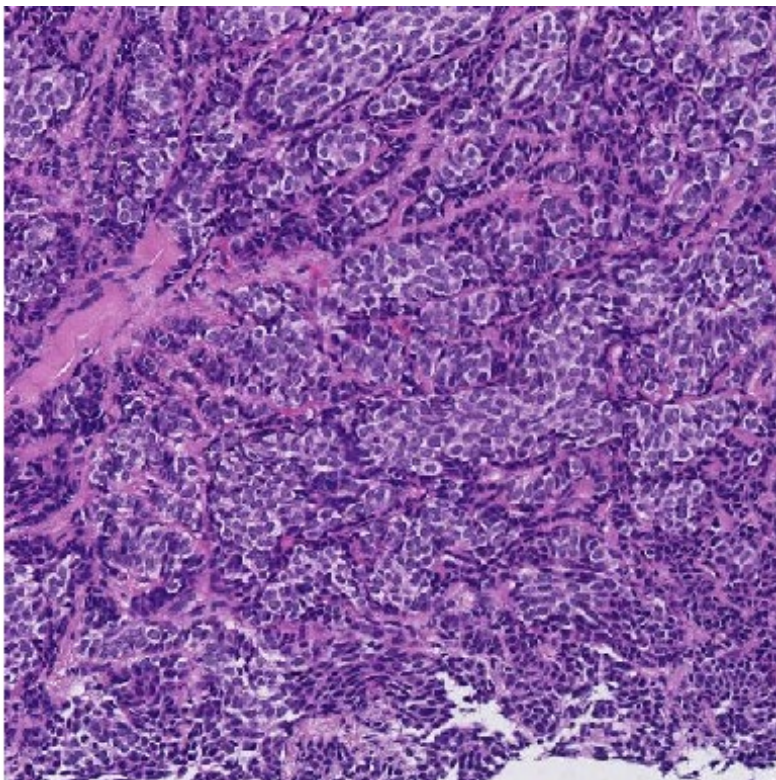
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	15	2
Evaluable	5	1
Evaluable	13	4
Evaluable	25	2
Evaluable	15	0
Evaluable	10	10

Mean Percent Tumor-Associated Stroma: 13.8

Mean sTILs Density: 3.2

Comments: There are no additional comments.

Pitfalls: There are no significant pitfalls for this ROI.



caseID: HTT-TILS-001-52B.ndpi_x71829.2190_y49452.2190

Expert Panel Annotations

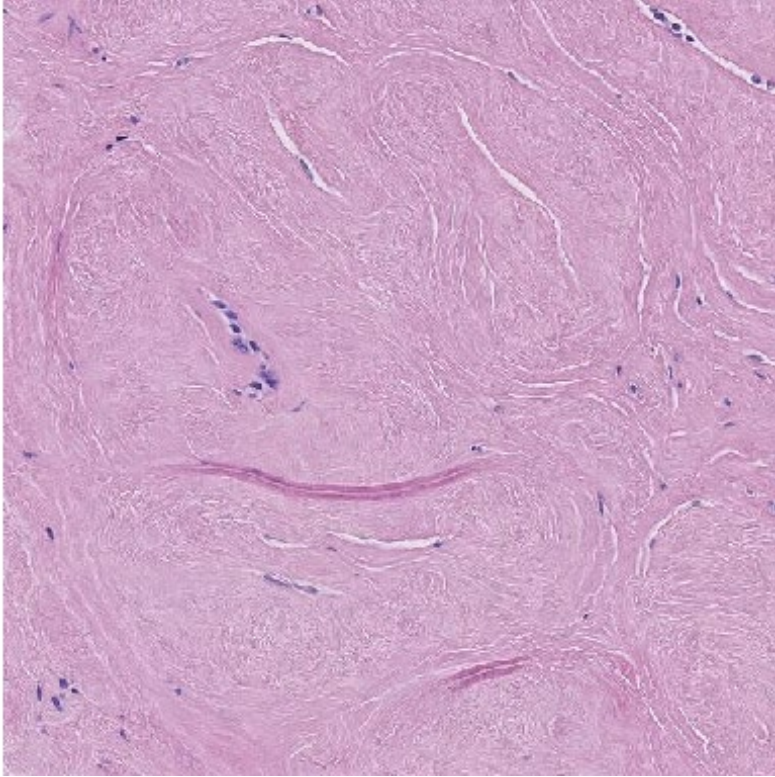
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	15	0
Evaluable	5	0
Evaluable	11	5
Evaluable	20	0
Evaluable	20	0
Evaluable	15	1

Mean Percent Tumor-Associated Stroma: 14.3

Mean sTILs Density: 1

Comments: Here, we see the invasive margin with 10% stroma and very sTILs. From an expert panelist, “this is actually a good case because all these very tiny dark round things, small round blue cells, are just probably a fixation artifact of ischemic tumor cells, and these are easily overlooked by machine learning process as being TILs.” At 8 o’clock, those dark cells are more likely tumor cells than lymphocytes and may be the result of preservation and/or staining artifacts.

Pitfalls: Non-lymphocytes may be confused for lymphocytes if there is tissue fixation artifact.



caseID: HTT-TILS-001-53B.ndpi_x28888.2190_y28271.2190

Expert Panel Annotations

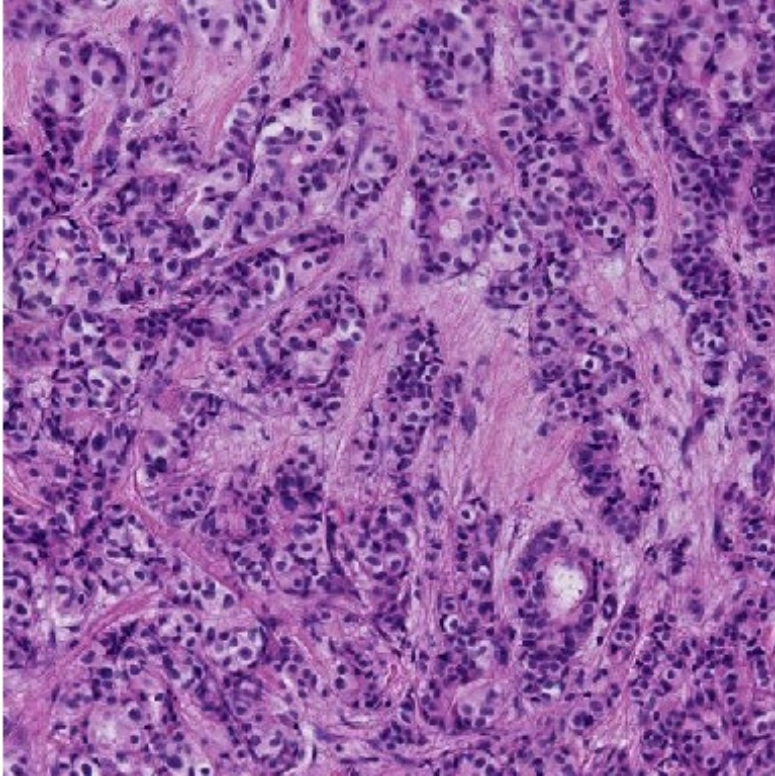
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Not Evaluable	NA	NA

Mean Percent Tumor-Associated Stroma: NA

Mean sTILs Density: NA

Comments: There are no tumor cells within the thumbnail of the ROI. Outside of the ROI beyond the lower right corner, we see fibrotic tissue and ducts, but it is unclear if there are tumor cells here.

Pitfalls: Invasive carcinoma must be present for sTILs density assessment.



caseID: HTT-TILS-001-57B.ndpi_x12938.2190_y7977.2190

Expert Panel Annotations

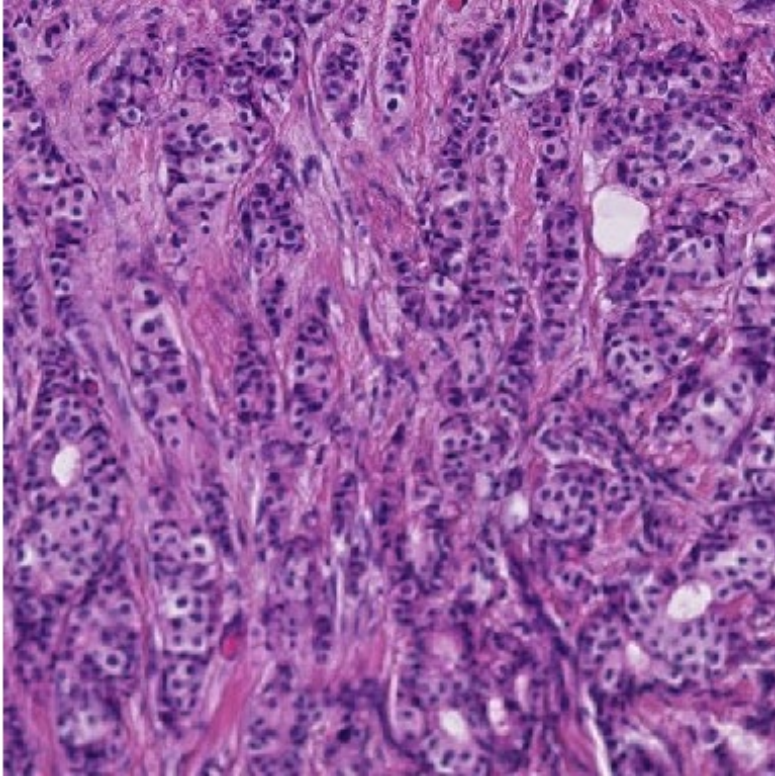
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	30	0
Evaluable	20	1
Evaluable	25	0
Evaluable	40	3
Evaluable	40	0
Evaluable	40	2

Mean Percent Tumor-Associated Stroma: 32.5

Mean sTILs Density: 1

Comments: There are no additional comments.

Pitfalls: There are no significant pitfalls for this ROI.



caseID: HTT-TILS-001-57B.ndpi_x16512.2190_y8168.2190

Expert Panel Annotations

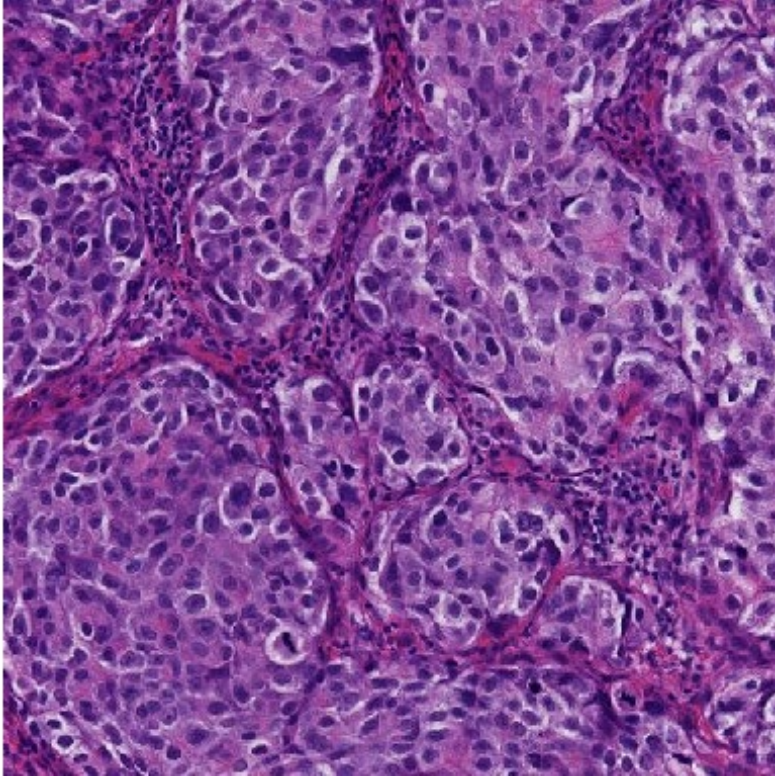
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	20	0
Evaluable	25	0
Evaluable	21	0
Evaluable	45	0
Evaluable	35	0
Evaluable	40	1

Mean Percent Tumor-Associated Stroma: 31

Mean sTILs Density: 0.2

Comments: There will be areas of out-of-focus because there are different thicknesses across the whole region.

Pitfalls: There will be areas of out-of-focus because there are different thicknesses across the whole region, or bad scan quality can contribute to an inaccurate assessment of TILs.



caseID: HTT-TILS-001-73B.ndpi_x9143.2190_y47362.2190

Expert Panel Annotations

ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	10	50
Evaluable	10	80
Evaluable	13	59
Evaluable	75	50
Evaluable	35	60
Evaluable	10	30

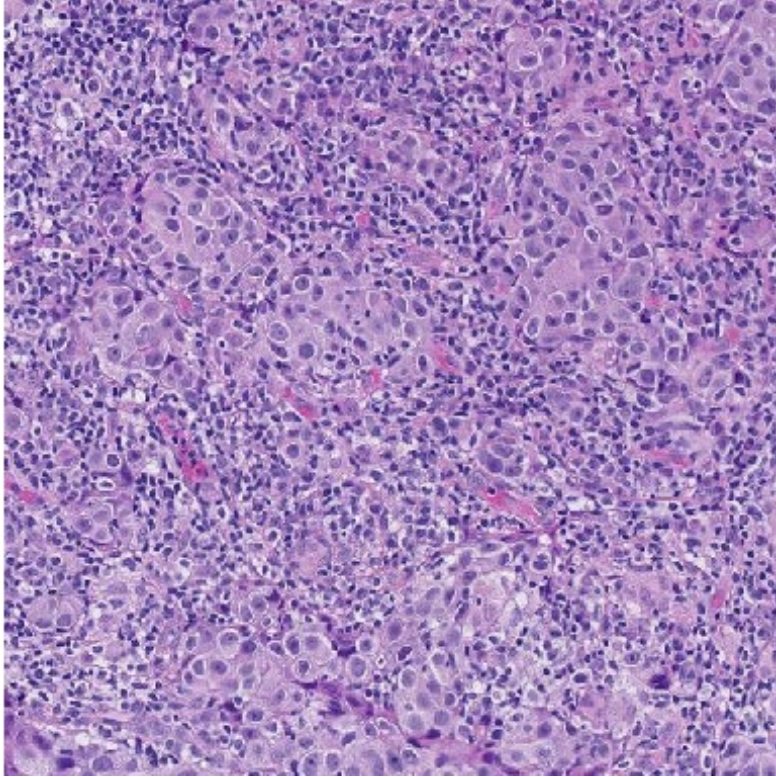
Mean Percent Tumor-Associated Stroma: 25.5

Mean sTILs Density: 54.8

Comments: In this slide, we have a dark H&E stain and can see about 5 axes of fibrous stroma in the ROI with 3 of them not having sTILs. The axes of fibrous stroma are challenging because it is unclear if this is all to be considered stroma, such as capillaries or other poorly visualized structures in the band. When there is not a lot of stroma present, it becomes harder to distinguish the density of TILs, but it is not likely to have the high density estimate of 80 sTILs in this ROI. One of the dogmas of sTILs scoring is that it is not

required for lymphocytes to have the same density as in a lymph node. It is okay for there to be space in between the lymphocytes.

Pitfalls: There are no significant pitfalls for this ROI.



caseID: HTT-TILS-001-76B.ndpi_x15338.2190_y44740.2190

Expert Panel Annotations

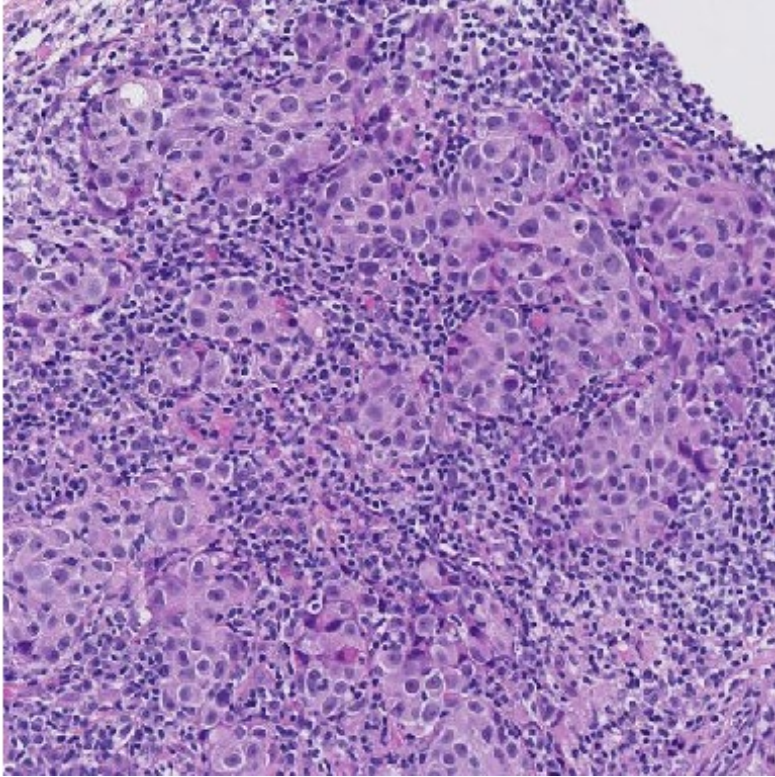
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	50	95
Evaluable	35	95
Evaluable	47	92
Evaluable	50	95
Evaluable	40	90
Evaluable	40	95

Mean Percent Tumor-Associated Stroma: 43.7

Mean sTILs Density: 93.7

Comments: There are no additional comments.

Pitfalls: There are no significant pitfalls for this ROI.



caseID: HTT-TILS-001-76B.ndpi_x16170.2190_y42769.2190

Expert Panel Annotations

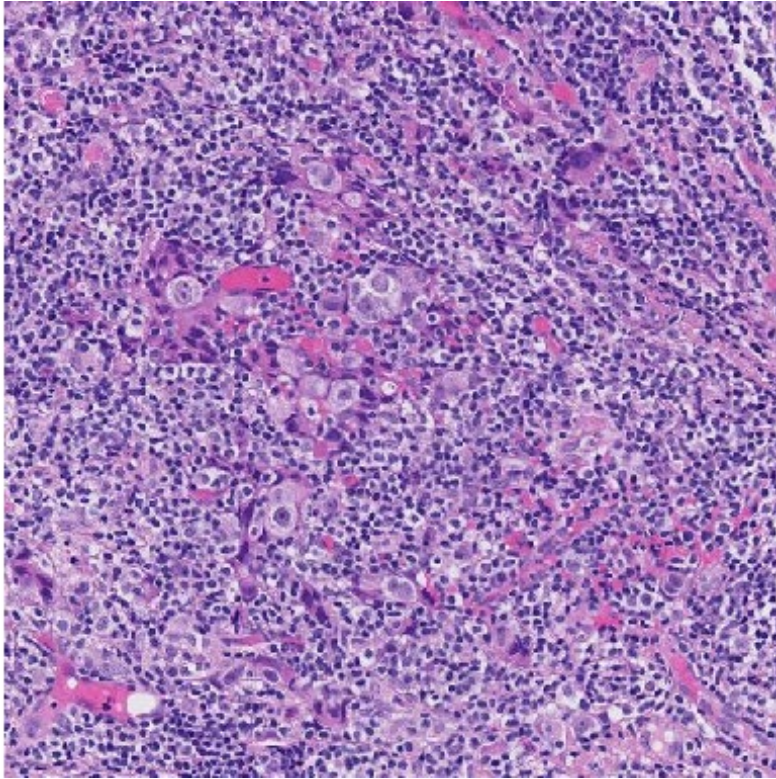
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	40	90
Evaluable	25	95
Evaluable	50	86
Evaluable	40	95
Evaluable	30	90
Evaluable	30	95

Mean Percent Tumor-Associated Stroma: 35.8

Mean sTILs Density: 91.8

Comments: When assessing sTILs, remember that the sTILs percentage is in proportion to the area of the tumor-associated stroma, not tumor cellularity or cell count percentage which will give you a different score. In sTILs assessment, the denominator is the area of tumor-associated stroma in the given ROI, and the numerator is the area of sTILs in that tumor-associated stroma.

Pitfalls: The percent of tumor-associated-stromal is calculated with respect to the area of the entire ROI. Negative/empty space is in the total ROI area, the denominator of the Percent Tumor-Associated Stroma.



caseID: HTT-TILS-001-76B.ndpi_x24500.2190_y76408.2190

Expert Panel Annotations

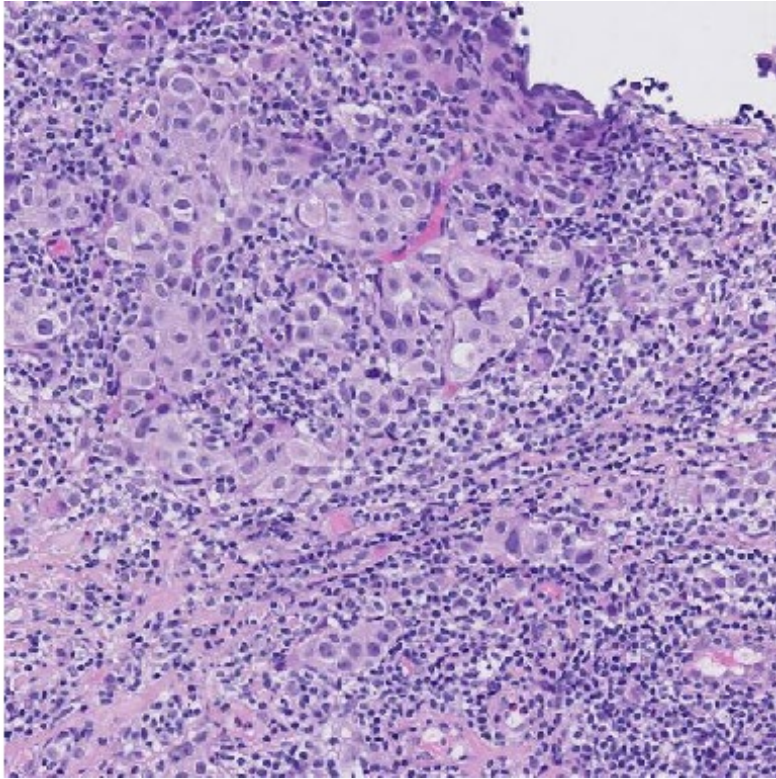
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	90	90
Evaluable	60	95
Evaluable	75	95
Evaluable	75	95
Evaluable	80	90
Evaluable	80	90

Mean Percent Tumor-Associated Stroma: 76.7

Mean sTILs Density: 92.5

Comments: This is intra-tumoral stroma with high stromal and sTILs densities.

Pitfalls: Perinuclear clearing can cause challenges in discrimination of cells, such as macrophages, lymphocytes/plasma cells, tumor cells, or others. Additional immunohistochemical staining may be helpful to further subclassify such cells. In regions where the sTILs density is very high, the underlying stroma may be obscured.



caseID: [HTT-TILS-001-76B.ndpi_x29068.2190_y50307.2190](#)

Expert Panel Annotations

ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	60	70
Evaluable	45	90
Evaluable	67	75
Evaluable	60	85
Evaluable	60	85
Evaluable	50	70

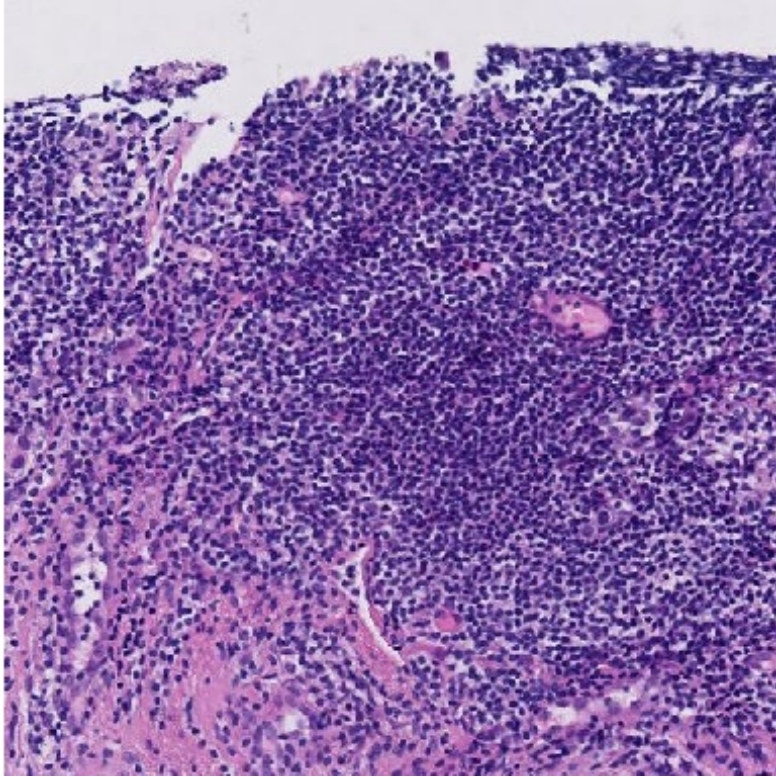
Mean Percent Tumor-Associated Stroma: 57

Mean sTILs Density: 79.2

Comments: There are 2 classical pitfalls demonstrated: crush zones (3 o'clock) and possible apoptotic tumor cells. Crushed cells become very dark and small, while apoptotic cells are round, blue cells. Both of which, if misinterpreted, can inflate the sTILs count. Fixation artifacts also give you this type of very small, once dark nuclei.

Pitfalls: Degenerating non-lymphocytes (e.g. pyknotic tumor cells) may be mistaken for lymphocytes. Non-lymphocytes may be confused for lymphocytes if there is tissue fixation

and/or cellular preservation artifact. Perinuclear clearing can cause challenges in discrimination of cells, such as macrophages, lymphocytes/plasma cells, tumor cells, or others. Additional immunohistochemical staining may be helpful to further subclassify such cells. In regions where the sTILs density is very high, the underlying stroma may be obscured.



caseID: HTT-TILS-001-80B.ndpi_x28631.2190_y33655.2190

Expert Panel Annotations

ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	90	90
Evaluable	90	90
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Evaluable	99	0
Not Evaluable	NA	NA

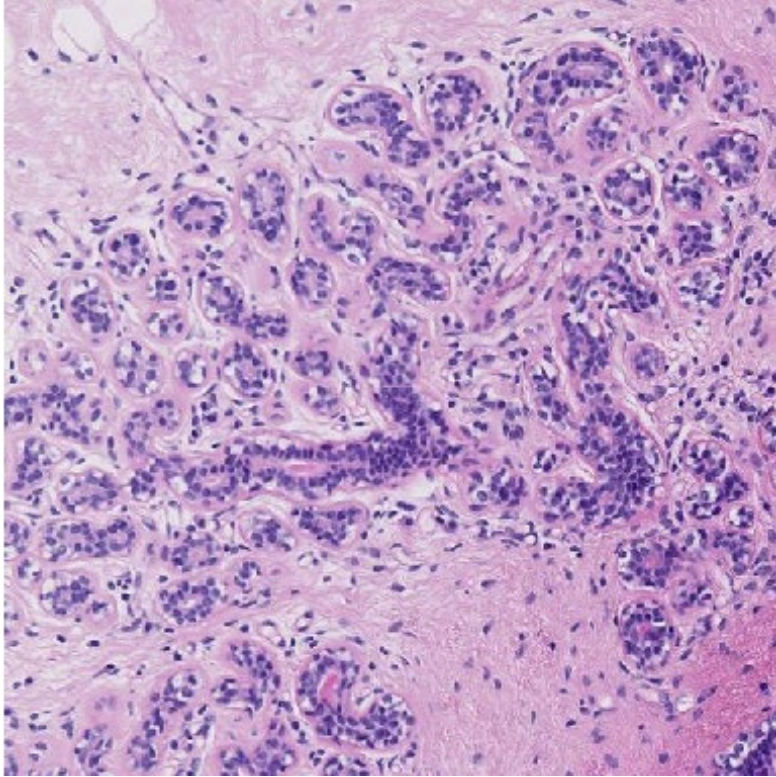
Mean Percent Tumor-Associated Stroma: 93

Mean sTILs Density: 60

Comments: A challenging case that highlights the limitations of core biopsies. It may appear that we are at the edge of tumor though we don't see an invasive margin. There is DCIS present to the right of this ROI, which should not be considered in sTILs evaluation. After discussion, group consensus was that this ROI is "Not Evaluable" for sTILs. This ROI might actually represent a lymphoid aggregate, which is not evaluated for sTILs. We suspect this is an aggregate because there is no stroma visualized between the TILs.

Making the distinction between a lymphoid aggregate and stromal TILs is a challenging task for AI/ML algorithms.

Pitfalls: DCIS should be excluded from the numerator when calculating the percentage of tumor-associated stroma. Invasive carcinoma must be present to perform the sTILs assessment.



caseID: HTT-TILS-001-80B.ndpi_x52173.2190_y57416.2190

Expert Panel Annotations

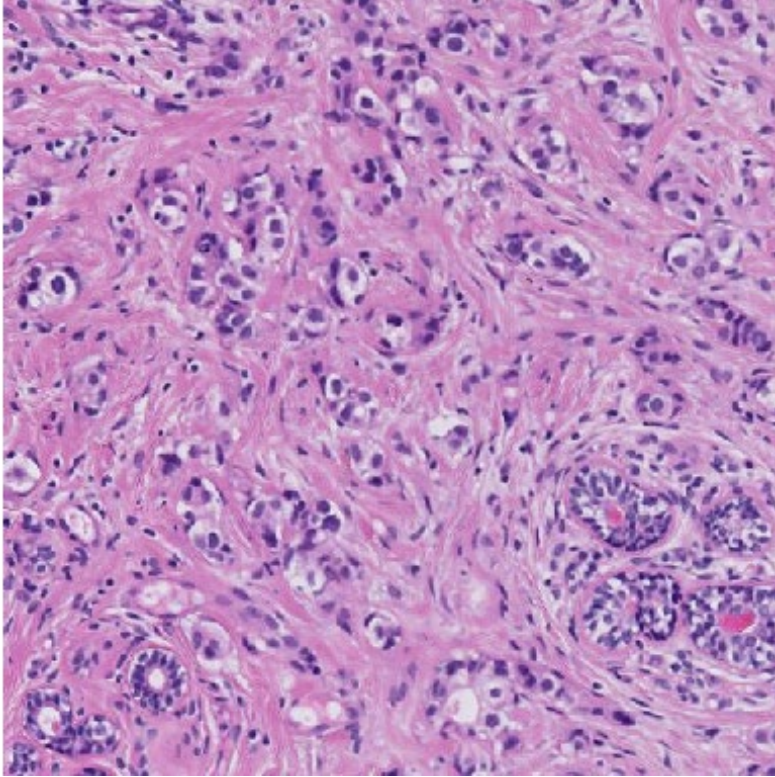
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Not Evaluable	NA	NA

Mean Percent Tumor-Associated Stroma: NA

Mean sTILs Density: NA

Comments: This is normal tissue.

Pitfalls: Invasive carcinoma must be present to perform the sTILs assessment.



caseID: HTT-TILS-001-80B.ndpi_x56330.2190_y11332.2190

Expert Panel Annotations

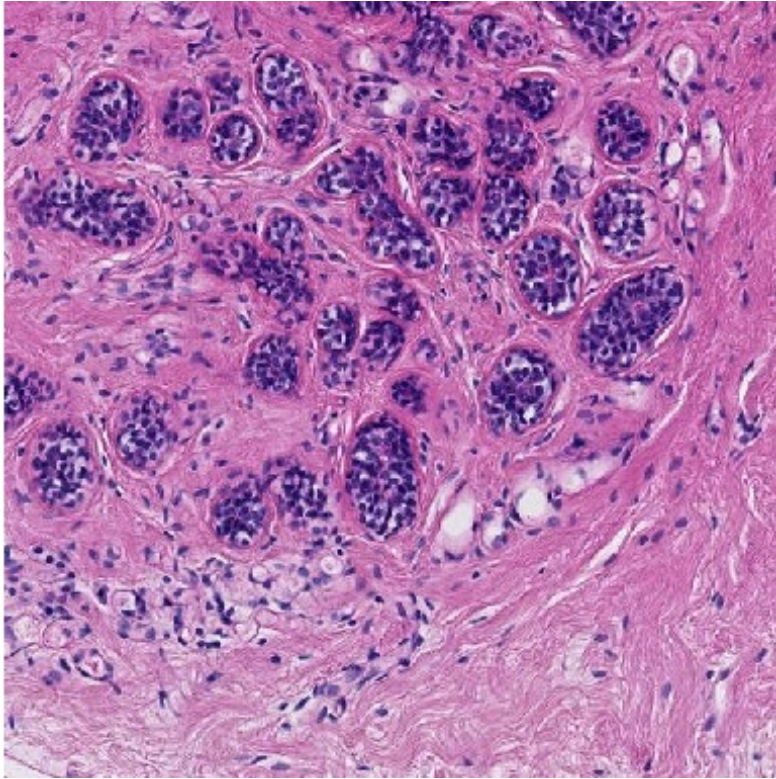
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	53	3
Evaluable	70	10
Evaluable	60	10
Evaluable	60	0
Evaluable	70	3
Evaluable	80	3

Mean Percent Tumor-Associated Stroma: 65.5

Mean sTILs Density: 4.8

Comments: In the center of this ROI, we can see fibroblasts that have been transversely sliced, which can be mistaken as lymphocytes. Most of the lymphocytes can be found in the upper left corner and fibroblasts can be seen in the 3 o'clock position where the fibroblasts appear slightly more elongated. Invasive carcinoma can be seen at the 9 o'clock and 10:30 positions with normal glands in bottom left (7 o'clock) and right (5 o'clock) corners.

Pitfalls: Axially sectioned fibroblasts may be mistaken for lymphocytes. DCIS and normal glands should be excluded from the numerator when calculating the percentage of tumor-associated stroma.



caseID: [HTT-TILS-001-80B.ndpi_x57261.2190_y59992.2190](#)

Expert Panel Annotations

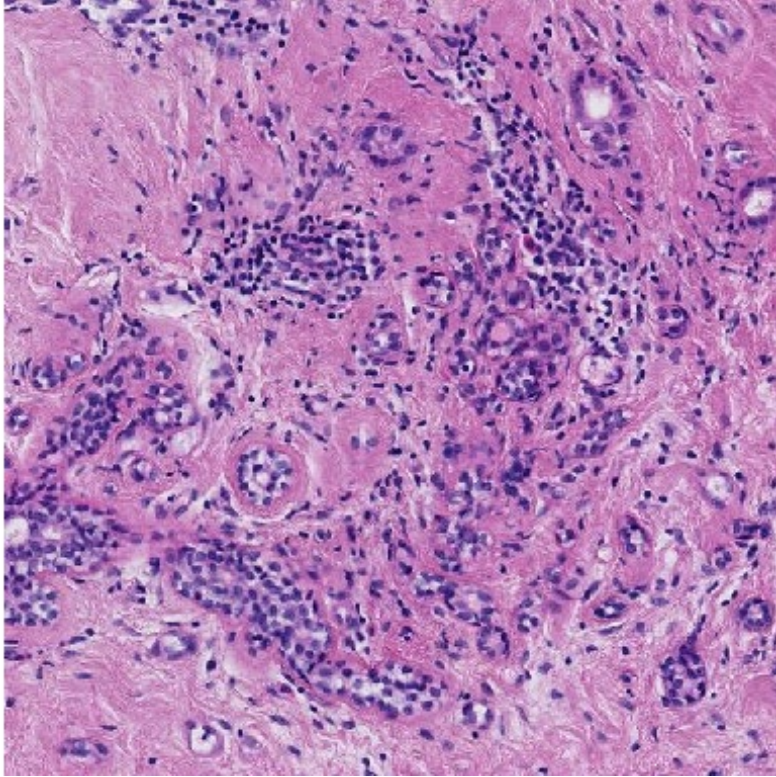
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Not Evaluable	NA	NA

Mean Percent Tumor-Associated Stroma: NA

Mean sTILs Density: NA

Comments: This is normal tissue.

Pitfalls: Invasive carcinoma must be present to perform the sTILs assessment.



caseID: HTT-TILS-001-83B.ndpi_x17750.2190_y20193.2190

Expert Panel Annotations

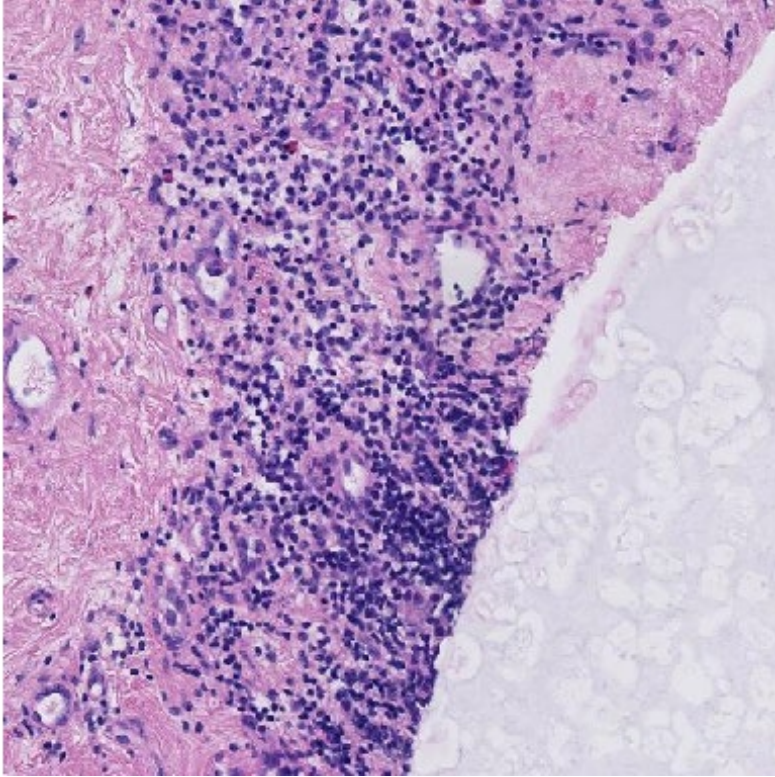
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Evaluable	75	5
Not Evaluable	NA	NA
Not Evaluable	NA	NA

Mean Percent Tumor-Associated Stroma: 75

Mean sTILs Density: 5

Comments: There are only 3 or so abnormal glands in this view that represent infiltrating tumor. However, it is expected to see lymphocytes in this ROI given the extent of normal tissue and scant tumor presence overall. This would not be an ROI to evaluate for stromal density. Any sTILs in this slide would have to be limited to the peri-tumoral stromal region which is minimal.

Pitfalls: Invasive carcinoma must be present to perform the sTILs assessment.



caseID: HTT-TILS-001-83B.ndpi_x24381.2190_y16599.2190

Expert Panel Annotations

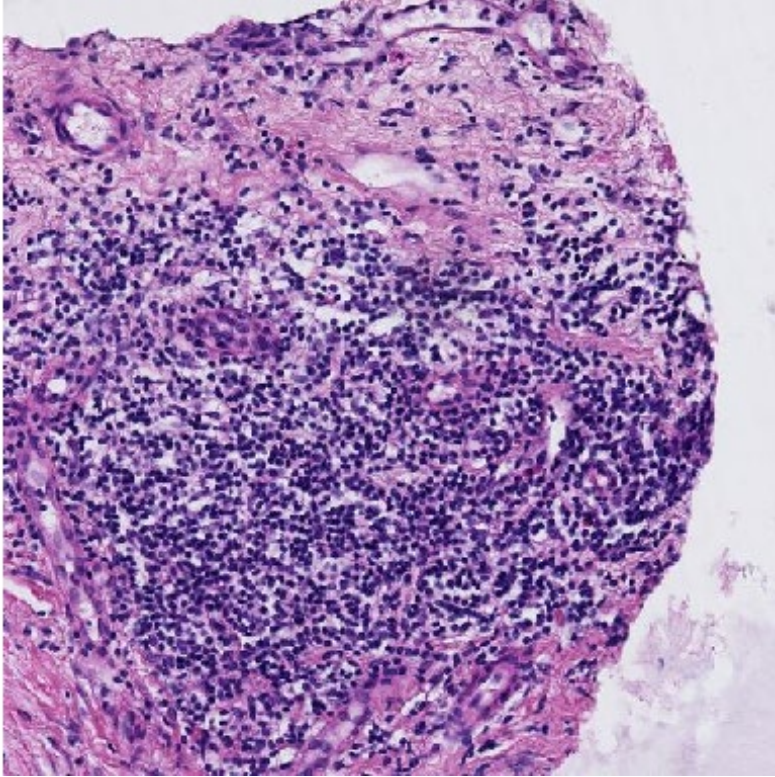
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Evaluable	56	0
Not Evaluable	NA	NA
Not Evaluable	NA	NA

Mean Percent Tumor-Associated Stroma: 56

Mean sTILs Density: 0

Comments: This is not tumor tissue.

Pitfalls: Invasive carcinoma must be present to perform the sTILs assessment.



caseID: HTT-TILS-001-84B.ndpi_x9416.2190_y29452.2190

Expert Panel Annotations

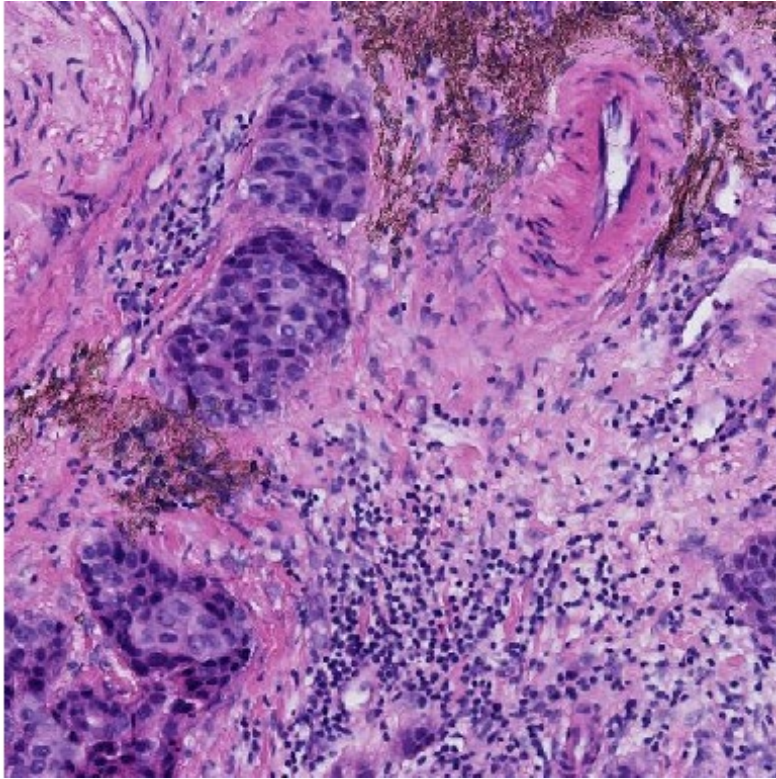
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Not Evaluable	NA	NA

Mean Percent Tumor-Associated Stroma: NA

Mean sTILs Density: NA

Comments: No invasive tumor cells present or adjacent to ROI.

Pitfalls: Invasive carcinoma must be present to perform the sTILs assessment.



caseID: HTT-TILS-001-86B.ndpi_x20669.2190_y12026.2190

Expert Panel Annotations

ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	75	30
Evaluable	35	60
Evaluable	86	15
Evaluable	75	30
Evaluable	70	25
Evaluable	70	20

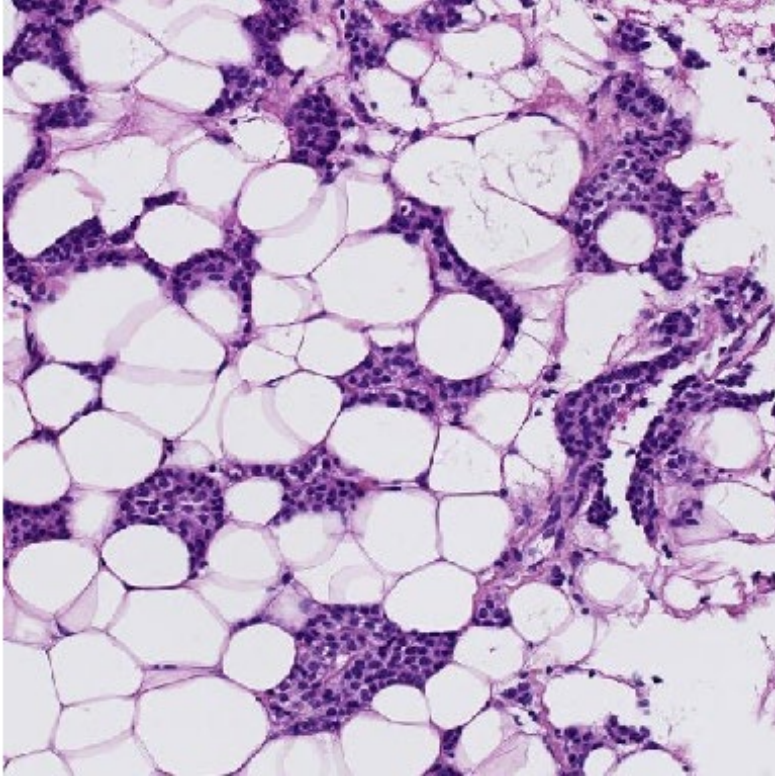
Mean Percent Tumor-Associated Stroma: 68.5

Mean sTILs Density: 30

Comments: There was high variability among our expert panel. The challenge in this case is the handling of the thick-walled vessel, determining if perivascular infiltration is present, and uncertainty of the structure in the upper left-hand corner of the ROI. This may present a challenge to pathologists when assessing sTILs in an ROI-based manner because the vessel would not have been considered when investigating a slide digitally or on a microscope. However, for a machine learning tool, it is important to define. Similar to infiltrating immune cells in adipose tissue, if there is no mesenchymal fibroblasts in

between there is not where for immune cells can bind to. “If we consider that reasoning that you don’t see TILs binding into the into the media of this vessel, we exclude it.”

Pitfalls: The walls of thick-walled vessels are not included as part of tumor-associated stroma.



caseID: HTT-TILS-001-88B.ndpi_x38756.2190_y32439.2190

Expert Panel Annotations

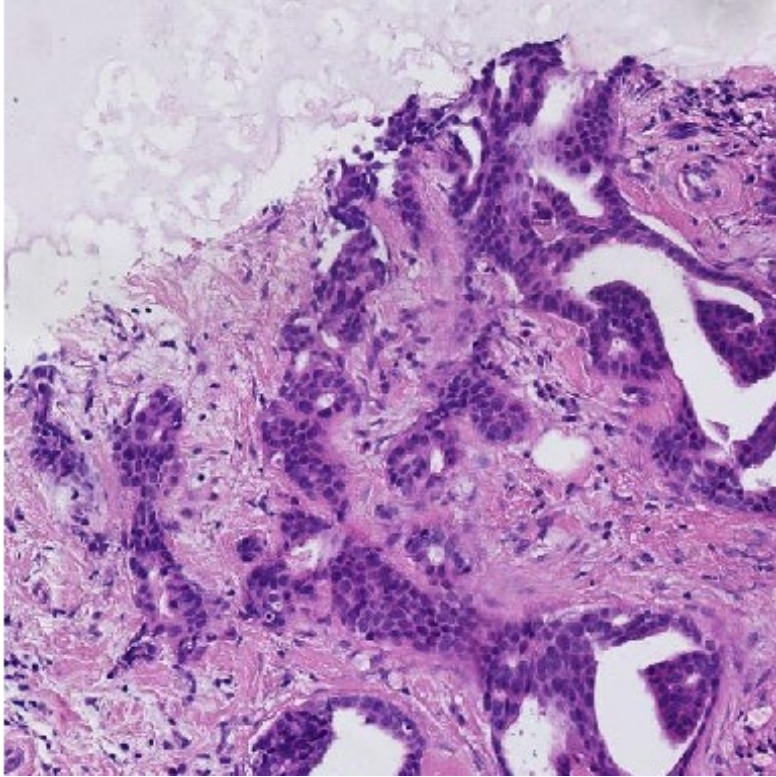
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Evaluable	4	0
Not Evaluable	NA	NA
Evaluable	10	0
Evaluable	20	1

Mean Percent Tumor-Associated Stroma: 11.3

Mean sTILs Density: 0.3

Comments: This ROI highlights the importance of understanding how evaluable stroma is defined. Though adipocytes may have biological significance in some scenarios, for the purpose of sTILs assessments, we don't consider them as tumor-associated stroma. Therefore, the amount of stroma between the adipose tissue in this ROI is low. Given the low-grade atypia, one may confuse the cancer cells as lymphocyte nuclei because they are very small and dark, but one may find a lymphocyte in the top right region of the ROI.

Pitfalls: Adipocytes are not considered part of the tumor-associated stroma for purposes of sTILs assessment. Non-lymphocytes with small nuclei may be confused for lymphocytes.



caseID: [HTT-TILS-001-89B.ndpi_x16220.2190_y7255.2190](#)

Expert Panel Annotations

ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Not Evaluable	NA	NA
Not Evaluable	NA	NA
Evaluable	33	8
Evaluable	50	5
Evaluable	60	1
Evaluable	50	5

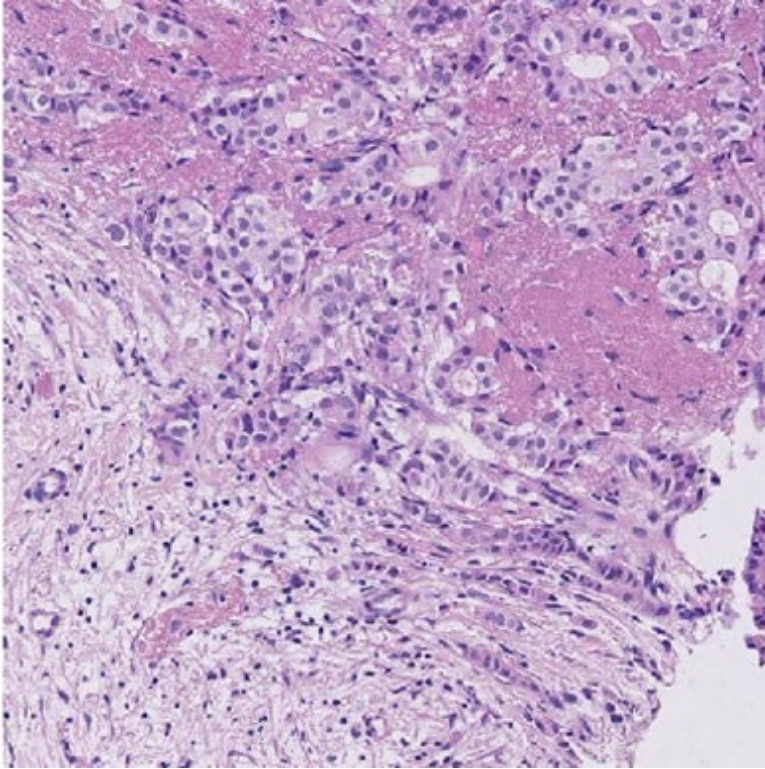
Mean Percent Tumor-Associated Stroma: 48.2

Mean sTILs Density: 4.8

Comments: Outside of the ROI, to the lower right, the tissue appears as invasive margin while DCIS can also be seen in the upper portion of the ROI. DCIS should not influence or be considered in the assessment of stromal percentage. In the upper right and 5 o'clock regions, the sTILs determination can be challenging due to possible perpendicularly cut fibroblasts. Another important consideration is the empty space must be considered when calculating the percent of tumor-associated stroma. Stromal percentage is calculated with respect to the area of the ROI and not with respect to the amount of tumor tissue present.

In the clinical setting, sTILs assessment is centered on the proportion of sTILs to tumor-associated stroma; the calculation of percentage of tumor-associated stroma is not performed. For the development of a reference standard for machine learning algorithms, the algorithm will calculate the percentage of tumor-associated stroma within a given ROI as part of its analysis. Percent Tumor-Associated Stroma is relative to the square, not the tissue.

Pitfalls: Axially sectioned fibroblasts may be mistaken for lymphocytes. DCIS should be excluded from the numerator when calculating the percentage of tumor-associated stroma. The Percent Tumor-Associated Stroma is calculated with respect to the area of the entire ROI. Negative/empty space is to be included in the total ROI area, the denominator of the Percent Tumor-Associated Stroma.



caseID: HTT-TILS-001-43B.ndpi_x12601.2190_y19050.2190

Expert Panel Annotations

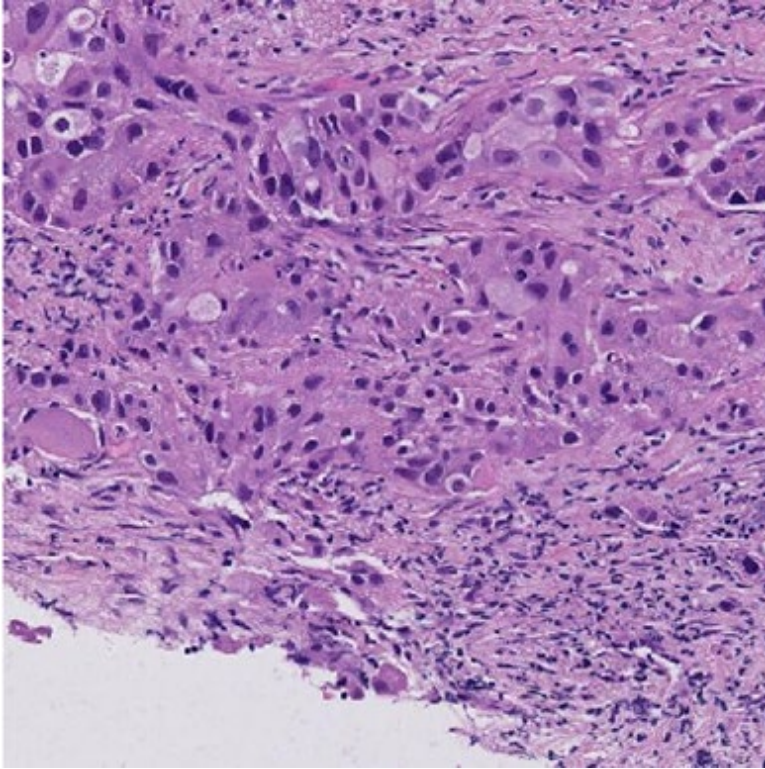
ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	65	5
Evaluable	70	15
Evaluable	65	10
Evaluable	30	20
Evaluable	40	10
Evaluable	60	5

Mean Percent Tumor-Associated Stroma: 55

Mean sTILs Density: 10.8

Comments: Stroma with hyalinization and elastosis are included, as long as invasive cancer cells are present, in the TIL assessment. Necrotic tissue is excluded.

Pitfalls: Necrosis is excluded from sTILs evaluation. Hyalinized and elastotic stroma are included in assessment if associated with invasive carcinoma.



caseID: HTT-TILS-001-50B.ndpi_x20589.2190_y28549.2190

Expert Panel Annotations

ROI Type	Percent Tumor-Associated Stroma	sTILs Density
Evaluable	35	30
Evaluable	50	20
Evaluable	35	20
Evaluable	35	20
Evaluable	31	41
Evaluable	40	25

Mean Percent Tumor-Associated Stroma: 37.7

Mean sTILs Density: 26

Comments: Crushed cells are difficult to assess in TILs, which are found in the lower-right hand corner between 3-6 o'clock. AI/ML algorithms may find it difficult to identify crushed lymphocytes.

Pitfalls: The Percent Tumor-Associated Stroma is calculated with respect to the area of the entire ROI. Negative/empty space is to be included in the total ROI area, the denominator or Percent Tumor-Associated Stroma. Non-lymphocytes may be confused for lymphocytes if there is cellular preservation artifact.

VII. References

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