# Double Staining Immunohistochemistry and Digital Pathology: Moving Towards Standardization of the Proliferative Index Evaluation in Meningiomas

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*Article*

# Double Staining Immunohistochemistry and Digital Pathology: Moving Towards Standardization of the Proliferative Index Evaluation in Meningiomas

**Viscardo Paolo Fabbri [1,*], Giuseppe Broggi [2,*], Giovanna Attolini [1], Vincenzo L'Imperio [3], Fabio Pagni [3], Angela Guerriero [4], Stefano Marletta[5], Francesco Fiorentino [6], Stefania Caramaschi [7,8], Claudia Malagoli [1], Albino Eccher [1,2,3,4,5,6,7] and Rosario Caltabiano [2]**

[1] Department of Laboratory Medicine and Anatomical Pathology, Pathology Unit, AOU Policlinico di Modena, Italy.
[2] Department of Medical and Surgical Sciences and Advanced Technologies "G.F. Ingrassia", Anatomic Pathology, University of Catania, Catania, Italy.
[3] Department of Medicine and Surgery, Pathology, University of Milano-Bicocca, IRCCS (Scientific Institute for Research, Hospitalisation and Healthcare) Fondazione San Gerardo dei Tintori, Monza, Italy.
[4] Department of Pathology Unit, Padua University Hospital, Padua, Italy.
[5] Division of Pathology, Humanitas Istituto Clinico Catanese, 95045 Catania, Italy.
[6] Pathology Unit, Santa Maria Goretti Hospital of Latina-ASL Latina, Latina, Italy.
[7] Section of Pathology, Department of Medical and Surgical Sciences for Children and Adults, University of Modena and Reggio Emilia, University Hospital of Modena, 41124 Modena, Italy.
[8] Clinical and Experimental Medicine PhD Program, Department of Biomedical, Metabolic, and Neural Sciences, University of Modena and Reggio Emilia, Modena, Italy.
* Correspondence: fabbri.viscardopaolo@aou.mo.it (V.P.F.); giuseppe.broggi@gmail.com (G.B.)

**Abstract:** *Background* Although several studies showed that high immunohistochemical Ki-67 index negatively influences the clinical course of meningiomas, especially in grade 1 and 2 neoplasm, the 5th edition of the World Health Organization (WHO) Classification of Tumours of the Central Nervous System does not recognize it as a prognostic criterion. Issues related to Ki-67 assessment include interobserver variability, different cut-off values used by pathologists and the presence of a complex inflammatory tumour microenvironment, that may overestimate the proliferative index (PI). *Methods* In this paper, we described as Double Staining Immunohistochemistry (dIHC) EMA/Ki-67 highlights only neoplastic meningocytes better tha single slices evcaluation; then the application of Digital Pathology (DP) can provide digital information for a more accurate count. *Results and Future prospective* dIHC and DP can support and standardize the evaluation of PI in meningiomas in daily routine.

**Keywords:** Meningioma; Double Staining Immunohistochemistry; Digital Pathology; proliferative index.

## 1. Introduction

Meningiomas are extra-axial tumours of the Central Nervous System (CNS) and account for 36.4% of all CNS neoplasms [1]. According to the 2021 WHO classification, meningiomas are stratified into grade 1 (benign), grade 2 (atypical) and grade 3 (anaplastic) subtypes [2]. This classification is primarily based on histological criteria: mitotic activity, brain invasion, solid growth, foci of spontaneous necrosis, hypercellularity, macronucleoli, high nuclear-cytoplasmic ratio of neoplastic cells and special morphologies (*i.e.* clear cell meningioma, chordoid meningioma and rhabdoid meningioma). Recently, molecular data may promote the development of an integrated classification with novel therapeutic approaches: for example, neoplasms with TERT mutation and CDKN2A/2B

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mutations exhibit a higher rate of malignant transformation, shorter time to recurrence, and lower overall survival (OS) [3].

The initial treatment for meningiomas (of all grades) is surgical resection [4]. Adjuvant radiotherapy (aRT) is the standard of care for grade 3 tumours, controversial for grade 2, and not indicated for grade 1.

Anaplastic meningiomas have a poor prognosis: median 5-year progression-free survival (PFS) and OS are 13.9 months and 56.9 months, respectively [5]. More variability is reported for grade 2 and grade 1 meningiomas: atypical meningiomas have a reported 5-year OS of 81%, with a recurrence rate (RR) of 28% [6]; for grade 1 meningiomas, RR from 7% to 20% is described (despite the OS is always >90%) [7], although gross total resection (GTR) could be performed, literature.

This different clinical behaviour leads to identify other prognostic parameters to improve therapeutical management after excision for those patients, dividing them in low-risk or high-risk progression.

The negative prognostic value of high Ki-67 proliferative index (PI) has been well-documented in most solid neoplasms [8–10] but the prognostic role remains unclear in meningiomas: nowadays, different cut-offs have been proposed by pathologists with high variation in the number of slices evaluated, fields selected, interpretation of neoplastic hotspots. In addition, difficulties can be found in excluding reactive microenvironment in a routinely performed Ki-67 single slice: lymphocytes, macrophages, endothelial cells in proliferative phase could be confuse with meningocytes and include in PI evaluation.

Different antibodies can evidence meningothelial cells (helping in differential diagnosis between meningioma and other tumours): they can divide into nuclear, cytoplasmatic and membranous markers. Among the nuclear antigens, Progesterone Receptor (PR) is expressed in 67.5% of grade 1, 66.6% of grade 2 and none of grade 3 neoplasms [11], whereas the most useful cytoplasmatic markers are Somatostatin Receptor type 2 (SSTR2) [12] and CD13 [13] (all described in up to 90% of tumours). EMA (Epithelial Membrane Antigen or MUC1) is characterized by membranous immunopositivity and it is the most used marker for the diagnosis of meningioma.

Aim of this paper is to evaluate PI in 20 non-consecutive meningiomas by an integrated use of Double Staining Immunohistochemistry (dIHC) EMA/Ki-67 and Digital Pathology (DP), in order to improve reproducibility and standardization of Ki-67 value.

## 2. Materials and Methods

This study includes 20 non-consecutive meningiomas (operated from January 2022 to December 2022): cases include 12 men and 8 women, ranging in age from 48 to 82 years old (median age 64).

Histological features, WHO grade and Ki67 (monoclonal antibody 30-9) were evaluated by four pathologists (VPF, GB, FF, GA).

A double-staining EMA (monoclonal antibody E-29) and Ki67 (monoclonal antibody 30-9) was performed in automated stainer (Ventana, Tucson, AZ, USA using purchased pre-diluted antibodies).

Procedure applied:

*First Stain*

Indirect immunohistochemistry with avidin-biotin peroxidase was performed with this protocol:

1. Wash twice in TBS 0.05M pH7.5, to which 0.01% Tween 20 has been added.
2. Briefly blot the slides without letting them dry and then apply 3% human or pig serum as a blocking agent (health hazard!).
3. Incubate with the blocking for 10 min. If one of your antibody is biotin-conjugated, you need at this point to do endogenous biotin blocking.
4. Blot the slides without washing and apply the primary antibody, in a moist chamber, at RT for 1-18 hr.
5. Wash twice in TBS 0.05M pH7.5 + 0.01% Tween 20.

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6. Add the biotin-conjugated secondary antibody (50 to 100 µl) and incubate for 45 min. The secondary antibody should be absorbed against human serum; if not add 1% human serum before use.

7. Wash twice in TBS 0.05M pH7.5 + 0.01% Tween 20.

7bis- block endogenous peroxidase by incubating in 0.1%$NaN_3$ and 0.3% $H_2O_2$ for 30 min. Wash thrice.

8. Add the HRP-conjugated avidin (50 to 100 µl, dilution 1:300 -500 in nTBS-Tween) and incubate for 20 min. Be careful not to dilute the avidin in biotin-containing medium.

9. Wash thrice in TBS 0.05M pH7.5 + 0.01% Tween 20.

10. Add 50 ml of the developing solution (see below ). Protect from direct light.

11. After 5 min, check the staining in your positive and negative controls.

12. Check the staining until complete, dense staining is obtained, but background is still low.

13. When staining is complete, wash thoroughly in tap water.

14. Transfer to TBS0.05M pH7.5 + 0.01% Tween 20.

*HRP Developing Solution:*

For 50 ml developing solution add in order:

- Aminoethylcarbazole (20 mg tablets, Sigma A-6926, dissolved in 2.5 ml NN-DM formamide)
- 50 ml acetate buffer pH 5.5 (52.5 ml of 0.1M acetic acid solution + 196.5 ml of a 0.1M Na acetate solution, bring to 500 ml)
- 25 µl $H_2O_2$ 30%.

Shake well.

Filter with a 45µm filter .

Keep away from direct light, use within 5 min.

*Second Stain:*

Double indirect immunohistochemistry was performed with this protocol:

1. Apply the 2nd primary antibody, in a moist chamber, at RT for 1-18 hr.
2. Wash twice in TBS 0.05M pH7.5 + 0.01% Tween 20.
3. Add the AP conjugated secondary antibody (50 to 100 µl) and incubate for 45 min. The secondary antibody should be absorbed against human serum; if not add 1% human serum before use. SBA Goat anti mouse AP or Goat anti Rabbit AP can be used 1:200 in TBS-BSA $NaN_3$.
4. Wash thrice in TBS 0.05M pH7.5 + 0.01% Tween 20.
5. Add the AP conjugated tertiary antibody (50 to 100 µl) and incubate for 15 min. The tertiary antibody should be absorbed against human serum; if not add 1% human serum before use. SBA Goat anti mouse AP or Goat anti Rabbit AP can be used 1:200 in TBS-BSA $NaN_3$.
6. Wash thrice in TBS 0.05M pH7.5 + 0.01% Tween 20.
7. Add 50 ml of the developing solution (see below ). Protect from direct light.
8. After 5 min, check the staining in your positive and negative controls.
9. Check the staining at 10-15 min interval.
10. When staining is complete (usually < 1 hr), wash thoroughly in tap water.
11. Preferably postfix in formalin for 4-5 hrs before mounting in water soluble mounting medium (glycerol gelatin).

Do not counterstain, unless you can afford a very gentle hematoxilyn hue in the nuclei.

*AP Developing Solution:*

For 50 ml developing solution add in order:

- 50 ml Tris-Hcl 0.1M pH 9.2 (1:10 from a stock solution 1M).
- Levamisole 1mM (12 mg).
- 20 mg Naphtol As BI phosphate (stock solution 40 mg/ml in NN-DM formamide, anhydrous, kept at -20°C).
- 10 mg Fast Blue BB Diazonium salt (Sigma F3378).

Shake well.

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Filter with a 45µm filter.

Keep away from direct light, use within 5 min.

Slides were then scanned at selectable magnifications by Deepinto slide scanner (by Menarini diagnostic s.r.l.).

With the advanced image viewer, it is possible to:

- change magnification (4x, 10x, 20x and 40x)
- use the zoom function
- use the focus slider to view different layers in Z-stack images
- manage the measuring, mark-up and text tools
- perform image analysis/apply image processing algorithms
- choose the file format for saving images.

The evaluation of PI obtained by the combination of dIHC/DP was then compare by the previous single Ki-67 at light microscope.

## 3. Results

This preliminary study includes 20 non-consecutive meningiomas (operated from January 2022 to December 2022): cases include 8 men and 12 women, ranging in age from 48 to 82 years old (median age 64).

Histological grades assigned were: twelve (12) grade 1, 7 (7) grade 2 and one (1) grade 3.

Despite the global comparison of PI by dIHC/DP and previous single slice count did not show any significative differences between values (*see Table 1*), some interesting and different approach to the PI evaluation should be highlighted.

- Pathologists can evaluate in the same slices a diagnostic (EMA) and a prognostic (Ki67) marker;
- In contrast to the single slice Ki-67 (Figure 1), dIHC detects two markers on the same section, helping to differentiate exactly proliferative neoplastic cells from non-tumoral microenvironment components: EMA identifies in brown the cytoplasm of meningothelial cells, whereas nuclear antigen Ki67 is red: only double-positive cells must be included in the PI count (Figure 2), whereas backgrounds elements, that are positive for Ki67 but not for EMA, must be excluded (Figure 3).
- digitalization of the slice allows the image acquisition and specific tools can measure the field selected (Figure 4), cells can be counted (Figure 5) and images can be virtually shared and stored. Distinct areas of 1 $mm^2$ were selected and 100 neoplastic cells were counted, highlighted and divided by macrophages, vessels, lymphocytes in a more precise resolution.

**Table 1.** Clinical data and Ki67 evaluation between Ki-67 in single slice and dIHC with DP.

| | Age | Sex | Site | Grade | Ki-67 | dIHC |
|---|---|---|---|---|---|---|
| Case 1 | 82 | M | Sphenoid bone | 1 | 8% | 7% |
| Case 2 | 47 | F | Parietal lobe | 1 | 7% | 7% |
| Case 3 | 64 | M | Sphenoidal bone | 2 | 9% | 11% |
| Case 4 | 62 | F | Parietal lobe | 1 | 7% | 6% |
| Case 5 | 58 | M | Frontal lobe | 1 | 7% | 5% |
| Case 6 | 66 | F | Temporal lobe | 2 | 7% | 9% |
| Case 7 | 71 | F | Frontal lobe | 1 | 7% | 7% |
| Case 8 | 66 | M | Frontal lobe | 3 | 25% | 29% |
| Case 9 | 50 | F | Sphenoid bone | 1 | 8% | 10% |
| Case 10 | 58 | M | Parietal lobe | 2 | 15% | 17% |
| Case 11 | 62 | F | Frontal lobe | 1 | 8% | 10% |
| Case 12 | 48 | F | Frontal lobe | 2 | 12% | 10% |
| Case 13 | 58 | F | Parietal lobe | 2 | 10% | 8% |
| Case 14 | 50 | M | Temporal lobe | 1 | 8% | 9% |
| Case 15 | 61 | M | | 1 | 9% | 10% |
| Case 16 | 63 | F | Parietal lobe | 1 | 10% | 12% |
| Case 17 | 55 | M | | 1 | 7% | 9% |

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| | | | | | | |
|---|---|---|---|---|---|---|
| Case 18 | 49 | F | Sphenoid bone | 2 | 6% | 5% |
| Case 19 | 50 | F | Sphenoid bone | 2 | 8% | 7% |
| Case 20 | 47 | F | Temporal lobe | 1 | 6% | 8% |

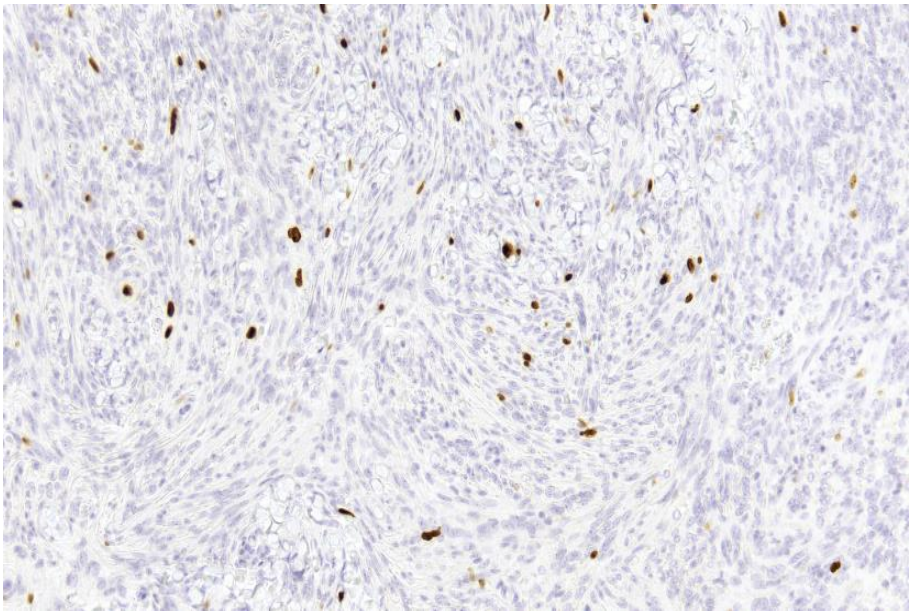

**Figure 1.** Example of single-slice Ki67. Nuclei in brown are in proliferative phase.

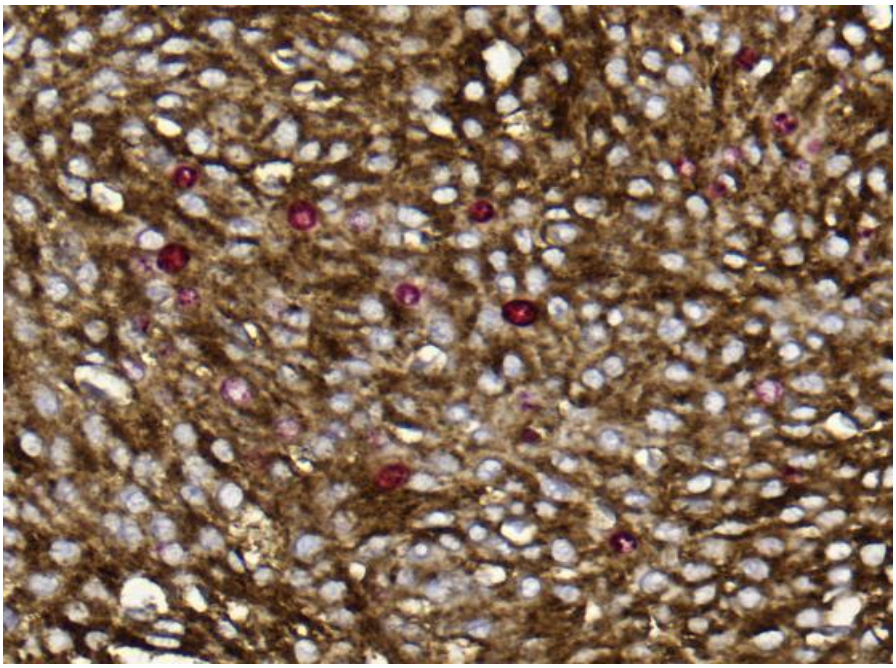

**Figure 2.** Example of dIHC. Meningocytes are brown (membrane) and Ki67 is red.

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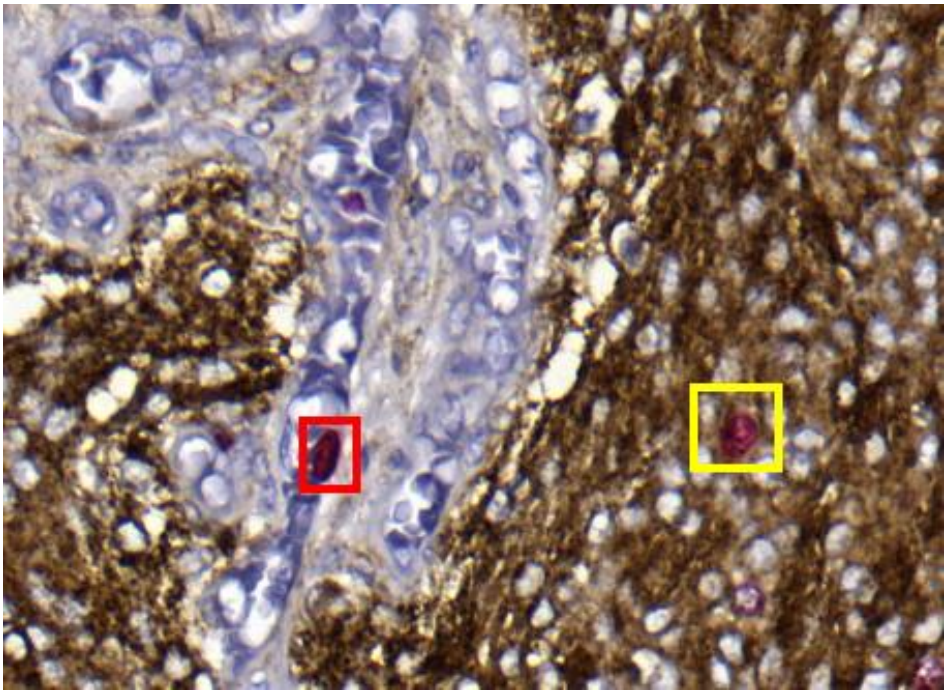

**Figure 3.** Only brown/red cells are proliferative meningocytes. Red cells alone can be microenvironment elements (in this case, endothelial cells and a little lymphocytes).

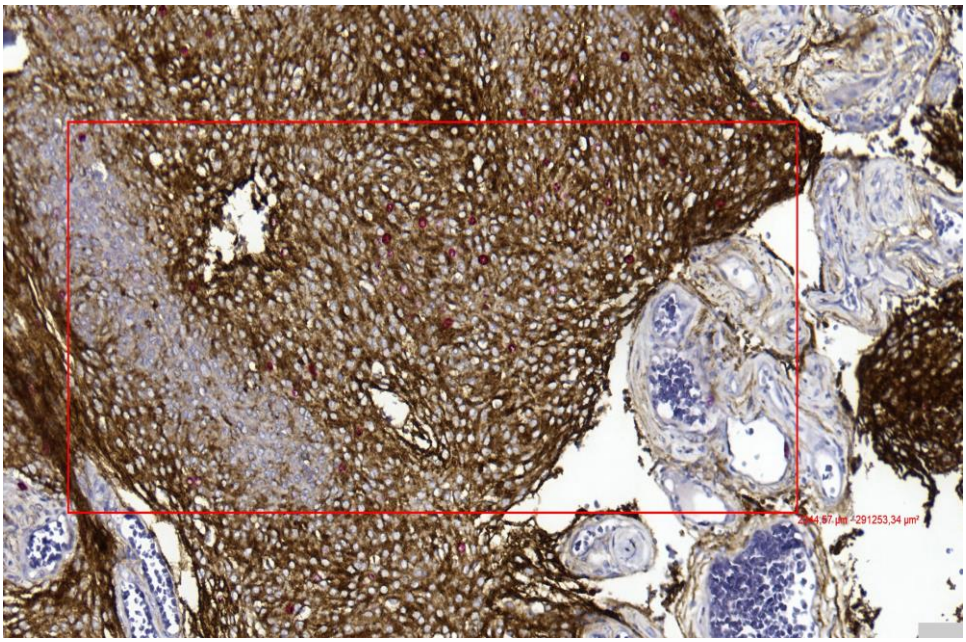

**Figure 4.** An example of area selection with a specific digital tool.

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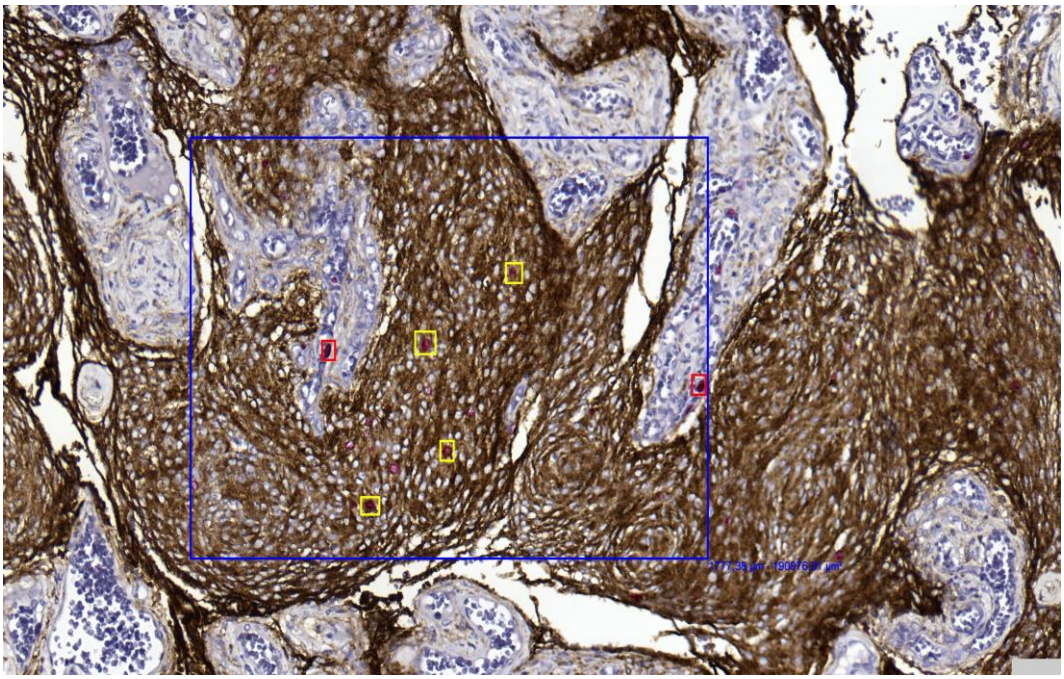

**Figure 5.** Digital tool selected the area and started to count proliferative neoplastic meningocytes (yellow) and proliferative microenvironment (red).

## 4. Discussion

Ki-67 protein (identified by Scholzer and Gerdes in the 1980s) is present during all active phases of the cell cycle (G1, S, G2, and M) but absent in resting cells (G0) [14]. Nuclear Ki-67 detection by immunohistochemistry (from formalin-fixed, paraffin-embedded tissue) is a well-proven test used by pathologists and it is associated with the proliferative index (PI) of malignant tumours, making it a marker of aggressiveness.

The role of Ki-67 in meningiomas has been investigated as a diagnostic, prognostic (for RR and OS), and predictive marker.

In daily practice, immunohistochemical assessment of Ki-67 can guide mitotic count and shows a consistent correlation with histological grade. In the study by Telugu et al. [15], meningiomas were distributed according to grade with arbitrary cut-off levels for Ki-67: PI for grade 1, grade 2, and grade 3 meningiomas were 3.1%, 7%, and 14.2%, respectively. Several studies suggest that tumours with Ki-67 >4% have RR like those of atypical meningiomas, while those with PI >20% behave like anaplastic meningiomas [16].

Retrospective studies and meta-analyses have shown that Ki-67 is a predictive marker for RR after surgery, especially in low-grade neoplasms. In WHO grade 1 meningiomas, a value greater than 6% has been strongly correlated with an increased risk of local recurrence following surgical resection. Nowak et al. observed that, among 535 consecutive patients who underwent surgical resection, recurrence was reported in 53 (17.7%) cases at a median follow-up of 31.5 months [17]. In a multivariate analysis by Lee et al., the Ki-67 index was the most powerful predictor of recurrence in atypical meningioma compared to tumour size, surgical excision using Simpson grade, and mitotic activity, with an RR of 25% [18]. Interestingly, Ki-67 prediction through a radiomic machine learning classifier revealed shorter PFS for meningiomas with Ki-67 levels ≥5% compared to tumours with Ki-67 <5% [19].

A recent meta-analysis involving 43 studies (5012 patients) found that higher Ki-67 expression levels were significantly associated with worse OS [14]. However, subgroup analysis revealed that factors like ethnicity, tumour grade, and cut-off values for heterogeneity investigation can affect the pooled results. Ki-67 may also be a predictive marker in the context of aRT for resected atypical and anaplastic meningiomas: 5-year PFS with Ki-67 ≤10% compared to >10% was 42.3% versus 20.0%,

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respectively. When stereotactic radiosurgery (SRS) was performed with a median prescription dose of 18 Gy, the 3-year local tumour control rates (LCRs) were 100%, 74%, and 25% for the low (Ki-67 <5%), intermediate (Ki-67 5-10%), and high (Ki-67 >10%) PI groups.

However, all reviews and meta-analyses highlighted several limitations: methodological differences in defining cut-off values (4% are defined arbitrarily) [14], the retrospective nature of studies with challenges in gathering clinical characteristics (e.g. size, grade, type of surgery), and the exclusion of non-English or unpublished studies lacking sufficient data. Therefore, more well-designed and large-scale prospective studies are recommended to confirm these findings.

In addition, because Ki-67 is generally evaluated on a single slice, the inflammatory background may overestimate the proliferative activity of neoplastic cells: reactive macrophages, lymphocytes, and endothelial cells are often immunopositive for Ki-67 but may be mistaken for meningothelial elements.

EMA (Epithelial Membrane Antigen or MUC1) is characterized by membranous immunopositivity and it is the most used marker for the diagnosis of meningioma. It is a transmembrane protein normally present in glandular or luminal epithelial cells, meningothelial cells or hematopoietic cells and its overexpression upregulates different inducers that lead to destabilization of junctions.

Despite in our small court Ki-67 are not significative different, a double-staining EMA (membranous) and Ki67 (nuclear) with the use of DP can give an aid in PI evaluation:

- dIHC immediately highlights with different chromogens only the proliferative meningothelial fraction in a single section (in contrast to single slice of Ki-67);
- DP can standardized the field or the cells counted (saving also pictures selected);
- slices can be share between pathologists (inside or outslide the laboratory);
- from a technical point of view, the use of dIHC reduces also the manual work of laboratory staff, the costs of sample preparation and the occupied space in pathology archive [20].

This study will lead to future research.

A larger cohort of meningiomas should be tested with dIHC and compare with the single slice Ki-67 immunoreaction, verifying the real advantages of this technique.

PI values should be then integrated with clinical, surgical, radiological data and artificial intelligence (AI) could be used to generate objective prognostic and predictive algorithms in order to predict low-risk or high risk (especially for grade 1 or 2 meningiomas).

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